STUDIES ON THE RESISTANCE OF COTTON, *GOSSYPIUM HIRSUTUM* L., TO BACTERIAL BLIGHT CAUSED BY *XANTHOMONAS MALVACEARUM*

I. PARTIAL PURIFICATION OF A PHYTOALEXIN PRODUCED IN Im 216 COTYLEDONS

II. INDUCED RESISTANCE IN Ac 44 COTYLEDONS

By

STEVE JOHN MCFAUL II

Bachelor of Science
College of Racine
Racine, Wisconsin
1973

Master of Science
Clemson University
Clemson, South Carolina
1976

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
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STUDIES ON THE RESISTANCE OF COTTON, GOSSYPIUM HIRSUTUM L., TO BACTERIAL BLIGHT CAUSED BY XANTHOMONAS MALVACEARUM

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II. INDUCED RESISTANCE IN Ac 44 COTYLEDONS

Thesis Approved:

Margaret Essenberg
Thesis Adviser

Ulrich Melcher

George V. Odell

Roger E. Koeppe

William M. Johnson

Norman N. Durham
Dean of the Graduate College

PREFACE

This thesis is divided into two sections: I. "PARTIAL PURIFICATION OF A PHYTOALEXIN PRODUCED IN Im 216 COTYLEDONS" (Pgs. 1 - 93); and II. "INDUCED RESISTANCE IN Ac 44 COTYLEDONS" (Pgs. 94 - 113). Section I investigates phytoalexin production in Im 216 cotton (Gossypium hirsutum L.) cotyledons which are immune to bacterial blight; Section II examines the possiblity of induced resistance in Ac 44 cotton (G. hirsutum) cotyledons which are normally susceptible to bacterial blight. The work presented in this thesis is a prelude to understanding specificity in the expression of host resistance genes.

I wish to thank Ellen Cover, Marlece Ebbesen and Paul Grover for their technical assistance and for their many hours of entertainment. In addition, their presence motivated me to finishing my research as quickly as possible. Also, I would like to thank Dr. William Johnson for providing Im 216 and Ac 44 seeds, the troughs and flats used for planting and for teaching me to respect high pressure sprayers. My appreciation also goes to Marcy Barron and to my other fellow members of St. John's folk group, "Spirit and Flesh", for their familial companionship and for giving me the chance to express myself in music. Special thanks go to my advisor, Dr. Margaret Essenberg, for her guidance and whose ambitious nature served as an inspirational model. Also, I would like to thank Judy Wollscheid for her support and understanding during the many rough times I experienced. Most importantly,

I wish to thank my parents, who mean everything to me, for their time, care and understanding during each and every day of my life.

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ALPHABETICAL LISTING OF ABBREVIATIONS

USED IN THE TEXT

Ac 44	Acala 44
AUFS	Absorbance units full scale
C.	Colletotrichum
cfu	Colony forming units
ChI	Challenge inoculum
G.	Gossypium
geq	Gram equivalent--the amount of phytoalexin present in a given gram weight of cotyledon tissue
H.	Helminthosporium
IC	Pertains to cotyledons inoculated with calcium carbonate
Im 216	Immune 216
IX	Pertains to cotyledons inoculated with X. malvacearum
n/n_c	$\frac{\text{Number of generations X. malvacearum undergoes in the presence of sample}}{\text{Number of generations X. malvacearum undergoes in the absence of sample}}$
P.	Phytophthora
PrI	Protection inoculum
Ps.	Pseudomonas
R3,R4	Race 3, Race 4
S.	Sclerotinia

Sp1,...SPn - SEP-PAK eluate where the number designates a percentage of methanol as described in the "RESULTS"

System I Hexane-ethyl acetate-methanol (60:40:1)

System II Chloroform-methanol (70:30)

X. Xanthomonas

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CHAPTER I

INTRODUCTION

Bacterial blight is a destructive disease of cotton which exists in most areas where the crop is grown; it is especially a problem where wind-driven rains or sprinkler irrigation disseminate the pathogen, Xanthomonas malvacearum (E. F. Sm) Dows., throughout the growing season (56,19). The long-stapled high quality tetraploid cottons of Gossypium barbadense L., which constitute an important percentage of the world crop, are highly susceptible to bacterial blight. In contrast, cultivars of G. hirsutum L., the major world cotton-producing species, exhibit varying degrees of resistance ranging from full susceptibility in Acala 44 (Ac 44) to total immunity in Immune 216 (Im 216) (19,18).

In Ac 44 leaves and cotyledons the disease appears as expanding water-soaked lesions which become chlorotic and within 2 to 3 weeks after infection become necrotic. The severity of the disease depends on the efficiency of the infection process; this is a function of climatic factors such as: wind-driven rainstorms, day and night temperatures, and relative humidity (56). Although death of the plant can occur under optimal infection conditions, there is a significant reduction in fiber yield even under the mildest of infection conditions (55).

In leaves and cotyledons of Im 216, however, inoculation with X. malvacearum elicits what is known as the "hypersensitive response." Two apparent consequences of the hypersensitive response are necrosis of

the invaded host tissue and inhibition of pathogen growth (40,54). The mechanism by which X. malvacearum growth is inhibited in Im 216 cotyledons is the subject of this research.

In 1940, Müller (65), from his work with different strains of Phytophthora infestans and tubers of resistant and susceptible potato hybrids, introduced his phytoalexin hypothesis: an antibiotic "principle", called a "phytoalexin", is formed by the host after infection as a direct result of contact with the pathogen and is responsible for inhibiting pathogen growth in hypersensitive tissue. I will refer to a phytoalexin, however, as a compound produced by the host, in response to infection, which has been found to be toxic in vitro to the invading pathogen. With this definition, a phytoalexin may or may not inhibit pathogen growth in hypersensitive tissue.

In host-pathogen systems other than cotton-Xanthomonas phytoalexins have been isolated (48,9,29,58). The present work investigates the possibility of phytoalexin production in Im 216 cotyledons as a mechanism of resistance to bacterial blight caused by X. malvacearum. It is the first aspect of a larger goal which is to understand, in chemical terms, the expression of specific genes for resistance: i.e., is differential resistance, which is conferred by various resistance genes in cotton against different races of X. malvacearum, expressed by production of the same inhibitor or of structurally different inhibitors? Knowledge of how these genes function will aid the plant breeder in selection of effective combinations of resistance genes as well as facilitate identification of resistance genes in the progeny. In addition, results of this work may provide a model for understanding disease resistance in other host-pathogen systems.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Description and Etiology

Bacterial blight of cotton was first described by Atkinson (3) in 1891; at that time, the disease was known as "Black Rust" of cotton. In 1892, however, Atkinson (4) published a fuller description of the disease in which the name had been changed to "Angular Spot" of cotton reflecting the characteristic angular spots which form on the foliage leaves. These spots originate as small water-soaked areas whose limits are determined by small anastomosing veins. A few lesions do not injure the plant to a great extent, but when they are numerous they coalesce to form large areas of dead tissue; the leaves soon wither and fall to the ground (71).

The causal agent of cotton blight, X. malvacearum, was first isolated from infected leaves and capsules (bolls) by E. F. Smith (73) in 1901. He described X. malvacearum as a gram negative, non-sporeforming, motile, rod-shaped schizomycete. It is strictly aerobic and produces a yellow polysaccharide slime. Smith (74) also was the first to reproduce the disease in healthy leaves by either spraying or gently rubbing inoculum onto the leaf underside. The primary means of infection is through the stomates; however, wounds from insects or other sources also provide a favorable place of entry (71). Once inside the plant,

X. malvacearum begins to grow within the intercellular space. When the intercellular spaces become filled with bacteria and slime the infected areas develop the familiar water-soaked appearance associated with the disease (63).

Disease Control

After discovery of X. malvacearum, measures were sought for control of the disease for which it was responsible, bacterial blight. Smith (74), in 1920, proposed the disease was transmitted by infected seed and Massey (63,64) confirmed this hypothesis through dissection of bolls from infected and noninfected plants. In addition, Massey (64) found that X. malvacearum was capable of long-term survival in dry cotton plant debris and that it was highly resistant to heat and dessication in this stage. He concluded that seasonal reappearance of the disease was largely due to carry-over of the pathogen from the previous year. Accordingly, efforts to control the disease have included disinfection of the seed surface by acid-delinting and removal of plant debris from fields after harvest.

Breeding for Resistance

In 1939, Knight and Clouston (55) initiated a breeding program for resistance. Through a series of crosses and backcrosses, they successfully transferred two major resistance genes from Uganda B31 (G. hirsutum) to the fully susceptible Sakel (G. barbadense) (55). The dominant resistance genes were termed "B_1" and "B_2" and their allelomorphs for susceptibility, "b_1" and "b_2", respectively. In either the homozygous or heterozygous state, B_2 confers greater resistance to Sakel

than does B_1. Knight and Clouston (55) also found that the resistance parent, Uganda B31, showed greater resistance than any progeny containing a $B_1B_1B_2B_2$ genome. They attributed the greater resistance to "modifying factors" or "minor genes" in Uganda B31 (55).

Knight and Clouston (55) were among the first to transfer major resistance genes from other Gossypium species into commercially acceptable strains. Since 1939, however, 17 B genes (oligogenes) have been identified for blight resistance (Table I). The resistance they confer is usually of the type which Van der Plank (81) described as "vertical"; it can be overcome only by specific races of the pathogen. Polygenic resistance, however, usually confers the same low level of resistance to all races of the pathogen; this is what Van der Plank (81) called "horizontal" resistance. Horizontal resistance is generally stable. Table II illustrates vertical and horizontal resistance with a set of host differentials and their genes for resistance.

The blight-immune line, Im 216, was developed from a heterozygous population of Bird's 101-102B strain using enforced inbreeding (20). Bird (17) synthesized the 101-102B strain by crossing Bar 4/16 (G. barbadense), which contained resistance genes B_2 and B_3, with Empire WR (G. hirsutum) containing the B_{Sm} minor gene complex. Several backcrosses to the recurrent parent, Empire WR, followed by selection of the most resistant plants of each segregating population, were required before immunity was detected (21). Immunity is defined here as "a response to all races of X. malvacearum which exhibits no macroscopic symptoms under field levels of inoculum" (21). Im 216, was selected from a segregating population of Birds 101-102B; homozygous immunity was obtained by selfing immune plants (21).

TABLE I

BLIGHT-RESISTANCE B GENES OF *GOSSYPIUM* SPECIES[1]

Gene Symbol	Source of Resistance
B_1	Uganda B31, a *G. hirsutum*, acclimatized in Africa.
B_2	Uganda B31 and other *G. hirsutum*: Punjab 513 Albar 49, Albar 51 UKBR Mebane B1, 17-3, CR4 Acala 1517 BR2, Acala 9136, Acala 8373; Blank & Hunter.
B_3	Schoeder 1306, an off-type *G. hirsutum* var. punctatum.
B_4	Multani strain NT 12/30 of *G. arboreum*.
B_5	Grenadine White Pollen, a perennial *G. barbadense*, probably with introgression from *G. hirsutum*.
B_6	Multani strain NT 12/30 of *G. arboreum*, probably also UKBR 61/12, a Tanzanian upland.
B_7	Stoneville 20, BBR-3 from Stoneville 2B, 8-3 from Half & Half.
B_8	*G. anomalum* (a noncultivated diploid species).
B_{9K}	Wagad 8, *G. herbaceum* (transferred by chromosome doubling).
B_{10K}	Kufra Oasis, *G. hirsutum* var. *punctatum*.
B_{9L}	Allen 51-296, *G. hirsutum*, Reba 296, *G. hirsutum*.
B_{10L}	Allen 51-296, *G. hirsutum*, Reba W296.
B_{11}	Wagad 8, *G. herbaceum*, (from same source as B_{9K}).
B_{In}	1-10B, *G. hirsutum*.

TABLE I (Continued)

Gene Symbol	Source of Resistance
B_N	20-8 and 20-3 from Northern Star, G. hirsutum.
B_S	6-77 from Stromproff No. 1, G. hirsutum.

[1]Taken from Brinkerhoff (19).

TABLE II

NINE RACES OF XANTHOMONAS MALVACEARUM AND SEVEN DIFFERENTIALS OF COTTON RANKED IN ORDER OF DECREASING MEAN VIRULENCE AND MEAN DISEASE SEVERITY[1]

	Disease Severity Index[2]							
Race	Ac 44	Ac B_2	OK 2.3	Ac b_7	Ac B_3	OK 1.2	Im 216	Mean
1	6.0	5.0	5.0	4.0	2.0	4.0	0.0	3.7
3	6.0	4.9	4.1	2.2	2.6	2.1	0.0	3.3
2	6.0	3.6	2.2	5.2	4.5	1.3	0.0	3.3
7	6.0	4.0	3.3	2.7	2.8	2.1	0.0	3.0
10	6.0	a	2.6	4.2	a	0.7	0.0	2.7
4	6.0	4.3	3.8	1.8	1.9	0.9	0.0	2.7
11	6.0	3.4	3.2	1.8	1.5	1.4	0.0	2.4
18	6.0	2.8	3.0	2.2	2.1	0.5	0.0	2.4
6	6.0	3.2	2.8	1.4	1.4	1.6	0.0	2.4
Mean	6.0	3.9	3.5	2.8	2.4	1.6	0.0	

[a]Not tested.

[1]Taken from Barnes (12).

[2]0.0 = No disease symptoms.
6.0 = Severe disease symptoms.

Im 216 has exhibited field resistance to all known races of the blight pathogen, X. malvacearum, since its development between 1960 and 1965 (William Johnson, personal communication). The apparent reason for this is the relationship between the B_2 B_3 genes and the B_{Sm} polygenic complex. As single genes in a susceptible background, they confer only low to moderate resistance; however, when coupled with each other or to polygenes, resistance is enhanced (19,56,21). Innes, et al. (45) suggested the B_{Sm} complex strongly modifies the B_2 B_3 genes forming a "supergene".

Expression of Resistance Genes

Genes for resistance of plants to microbial pathogens are expressed in various ways. Bell (13) has classified these in terms of anatomical barriers, nutritional limitation, resistance to the pathogen's enzymes or toxins and production of antimicrobial compounds. They may involve preformed or induced factors (54,28). Resistance of sugarcane to eyespot disease is an example of preformed resistance. In 1973, Strobel (79) showed that Helminthosporium sacchari, the eyespot pathogen, produces the phytotoxin helminthosporoside (2-hydroxy-cyclopropyl-α-D-galactopyranoside) which binds to a membrane protein of susceptible clones, but not to those of resistant clones. Strobel (79) found this was due to differences in four amino acid residues of the membrane proteins from susceptible and resistant clones.

A common induced form of resistance is the hypersensitive response. Upon invasion, resistant host cells in contact with the invading pathogen quickly die, and spread of the infection is prevented (54). With bacteria, it has been shown for a few cases that the num-

ber of necrotic lesions produced is directly proportional to the number of bacterial cells introduced into the host (51,62,80,32). It has also been shown, in tobacco and in cotton, that all concentrations of inoculum induce a hypersensitive response (54,80,32). Confluent necroses (massive cell death) is observed in tobacco leaves which have been inoculated with $\geq 5 \times 10^6$ cells of Pseudomonas pisi, a pathogen of pea; whereas concentrations $< 5 \times 10^6$ cells/ml lead to cellular death which could only be seen with a microscope. Imune 216 cotton leaves respond similarly to various concentrations of X. malvacearum (32).

Hypersensitivity and Phytoalexin Production

The interaction between Ps. pisi and tobacco leaves is an incompatible one since disease symptoms do not occur. Growth of the pathogen within an incompatible host is inhibited shortly following inoculation. In contrast, bacterial multiplication within the compatible host continues and disease symptoms develop (54,32,69,44,76,52,88,66). Since growth of a pathogen is inhibited only within an incompatible host, it seems likely that the inhibition is associated with the hypersensitive response of the host. In support of this idea was the work of Gnanamanickam and Patil (38). They found that if they treated bean plants with phaseotoxin, the chlorosis-inducing extracellular toxin produced by Ps. phaseolicola, prior to inoculation with an incompatible strain of Ps. phaseolicola, the hypersensitive response was suppressed and normal disease symptoms occurred. Furthermore, bacterial multiplication in toxin-treated plants was greater than in hypersensitivily responding controls. Thus suppression of the hypersensitive response reduces the ability of an incompatible host to inhibit growth of the

pathogens.

Phenolic compounds have been found to accumulate in the tissues of hypersensitivily reacting plants (28). Many of these and other compounds have been found to be toxic to both fungal and bacterial pathogens. Müller (65) termed any compounds which are produced by the host in response to infection and which are toxic to the invading pathogens, "phytoalexins". One of the earliest phytoalexins was detected in 1943 by Hiura (43) in sweet potato roots (Ipomaea batatas Lam.) infected with Ceratocysts finalriata. This compound was later identified by Kubota and Matsuura (57) as ipomeamerone (see Table III).

Since then, phytoalexins have been isolated from a variety of host tissues in response to fungal, bacterial and viral infections. In 1958, Müller (65) demonstrated the presence of a phytoalexin produced in bean pods (Phaseolus vulgaris) during its necrotic response to either the fruit pathogen, Sclerotinia fructicola or the potato pathogen, Phytophthora infestans. In addition, Müller (65) found the active material to inhibit the development of five other fungal plant pathogens not used for inoculation: Colletotrichum lindemuthianum, Uromyces trifolii, Botrytis cinerea, Pythium ultimum and Rhizoctonia solani.

Although Müller (65) had thoroughly characterized this phytoalexin in terms of its chemical properties, light sensitivity, time of production and output in relation to the age of the pods, it was not until 1964 that its structure was determined by Perrin (67). The phytoalexin was named "phaseollin" and was classified as a pterocarpan (see Table III). In 1971, Stholastuta, et al. (76 showed that, in addition to stimulation by fungal pathogens, phaseollin production in bean leaves was also elicited following inoculation with an incompatible strain

TABLE III

A CHRONOLOGICALLY ORDERED COMPILATION OF SOME PHYTOALEXINS ISOLATED SINCE 1943[1]

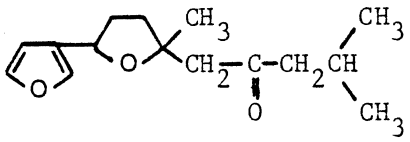
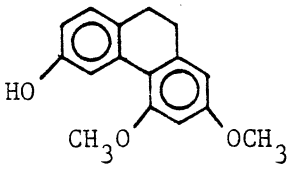
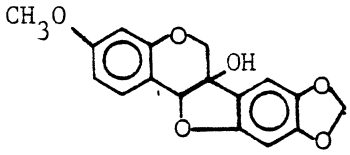
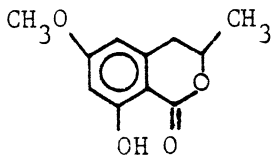
Phytoalexin	Source	Reference
 Ipomeamerone	Sweet Potato Roots (Ipomea batatas Lam.)	43,57
 Orchinol	Orchid Tubers (Orchis sp.)	35,36, 41
 Pisatin	Pea Pods (Pisum sativum)	25
 Substituted Isocoumarin	Carrot Roots (Daucus carota)	23,24

TABLE III (Continued)

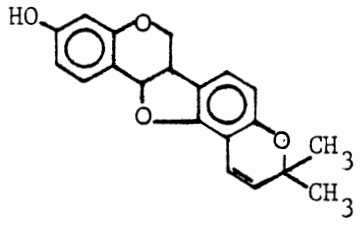
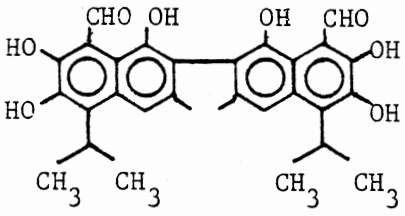
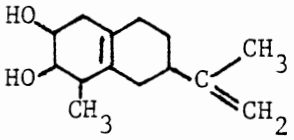
Phytoalexin	Source	Reference
 Phaseollin	Bean Pods (Phaseolus vulgaris)	67,68
 Gossypol	Cotton Glands (Gossypium hirsutum)	11,78 14
 Rishitin	Potato Tubers (Solanum tuberosum)	46

TABLE III (Continued)

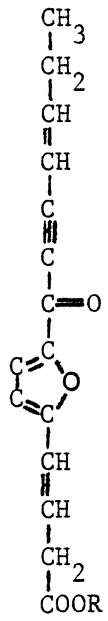
Phytoalexin	Source	Reference
		
R = H Wyerone Acid	Broad Bean	
R = CH_3 Wyerone Methyl Ester	(Vicia faba)	59,34
$C_{17}H_{26}O_4$	Potato Tuber	
Phytuberin	(Solanum tuberosum)	82

TABLE III (Continued)

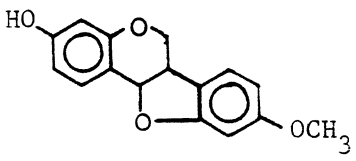
Phytoalexin	Source	Reference
 Safynol	Sunflower (Helianthus esp)	1
 Medicarpin	Alfalfa (Medicago sativa)	72

TABLE III (Continued)

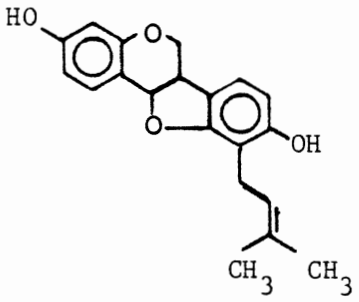
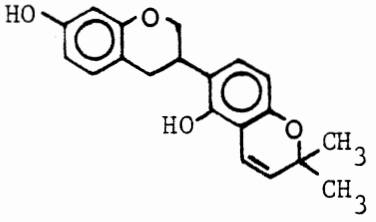
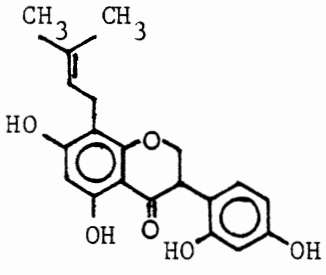
Phytoalexin	Source	Reference
 Phaseollidin	Bean Hypocotyl (Phaseolis vulgaris)	68,8
 Phaseollinisoflavan	Bean Hypocotyl (Phaseolis vulgaris)	8
 Kievitone	Bean Hypocotyl (Phaseolis vulgaris)	8

TABLE III (Continued)

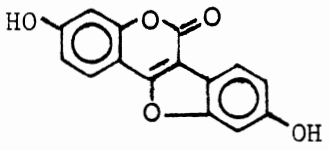
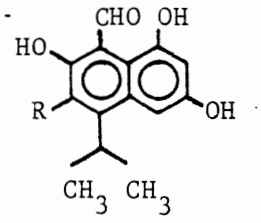
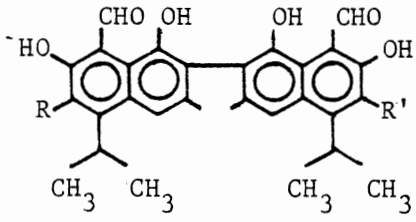
Phytoalexin	Source	Reference
 Coumestrol	Bean Leaves (*Phaseolis vulgaris*)	61
 R = OH Hemigossypol R = OCH_3 6-Methoxyhemigossypol R = H 6-Deoxyhemigossypol	Cotton Root and Stele Tissue (*Gossypium hirsutum*)	16,14
 R = OCH_3, R' = OH 6-Methoxygossypol R = R' = OCH_3 6,6'-Dimethoxygossypol	Cotton Root and Stele Tissue (*Gossypium hirsutum*)	78,14

TABLE III (Continued)

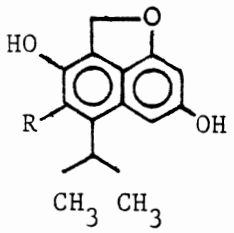
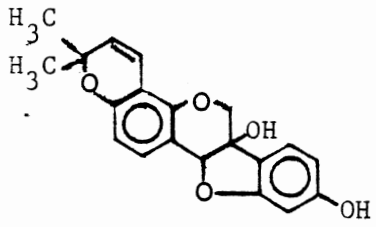
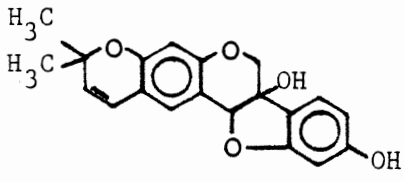
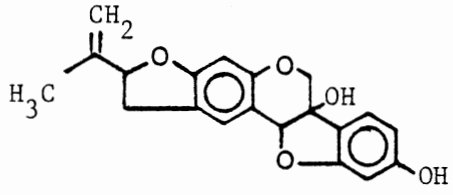
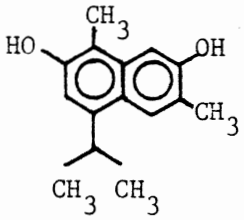
Phytoalexin	Source	Reference
		
R = OH Desoxyhemigossypol		
R = H Desoxy-6-deoxyhemigossypol	Cotton Stele Tissue	
R = OCH_3 Desoxy-6-methoxyhemigossypol	(Gossypium hirsutum)	77,14
		
Glyceollin I	Soybean (Glycine max)	49,22
		
Glyceollin II	Soybean (Glycine max)	60

TABLE III (Continued)

Phytoalexin	Source	Reference
 Glyceollin III	Soybean (Glycine max)	60
 2,7-Dihydroxycadalene	Cotton Cotyledons and Leaves (Gossypium hirsutum)	33

[1]Also see references 29, 58 and 47

of Ps. phaseolicola, a bacterial pathogen of bean. Phaseollin, however, at levels as high as 100 µg/ml had no effect on growth of this bacterium in culture. Wyman and Van Etten (87) found phaseollin inhibitory to only a few isolates of Ps. phaseolicola. Lyon and Wood (61) found that phaseollin did not inhibit growth of the fruit pathogen, Ps. morsprunoreum. In contrast, Gnanamanickam and Patil (37) found phaseollin to be inhibitory toward the growth of Ps. phaseolicola on solid media; and Gnanamanickam and Smith (39) found it much more inhibitory to gram-positive than to gram-negative bacteria. Thus, the role of phaseollin in resistance of bean to bacterial pathogens is at present uncertain. Other phytoalexins toxic to bacterial pathogens, however, have since been reported to accumulate in bean leaves inoculated with either fungi, bacteria or viruses (61,8,7,37). These are phaseollin, phaseollinisoflavan, coumestrol and kievitone.

Following Müller's (65) work and the subsequent identification of phaseollin, investigators began searching for phytoalexin production in other plants. Cruickshank and Perrin (25), in 1960 found that pea pods (Pisum sativum) inoculated with spore suspensions of S. fructicola produced a phytoalexin they identified as pisatin (Table III). It was later found that pisatin production was induced by at least 19 other fungi and spore-free germination fluids (58). Two phytoalexins, rishitin and phytuberin, have been isolated in resistant potato tubers undergoing a hypersensitive response to P. infestans (58,46,82). Klarman, et al. (48,49) isolated from soybeans inoculated with an incompatible strain of P. megasperma var. sojae, an antifungal agent with spectrophotometric properties similar to those of phaseollin. Keen, et al. (47) proposed a chemical structure for this phytoalexin which

they named 6-a-hydroxyphaseollin and demonstrated through mass spectrometry and gas-liquid chromatography that it existed as three isomers. Burden and Bailey (22) later revised the structure of 6-a-hydroxyphaseollin and it is now known as Glyceollin I (see Table III). The structures of the other two isomers, Glyceollin II and Glyceollin III, were identified in 1976 by Lyne, et al. (60).

In cotton, Bell, et al. (11,12,77,78,16) have identified a variety of terpenoids which are toxic to the wilt organism, Verticillium dahliae. They are: gossypol, 6-methoxygossypol, hemigossypol, 6-methoxyhemigossypol, 6-deoxyhemigossypol, desoxyhemigossypol, desoxy-6-methoxyhemigossypol (Vergosin) and 6,6'-dimethoxygossypol. These terpenoids are found in different ratios in various tissues of the cotton plant; their levels are a function of the age of the plant and increase following infection with the fungal pathogens, Verticillium dahliae, Rhizoctonia solani and Fusarium oxysporum (14,15). Before infection, each of the terpenoids is restricted to pigment glands.

Since the introduction of the "phytoalexin" theory by Müller (65) in 1940, the importance of phytoalexins in disease resistance has been debated (58). Although phytoalexins have been shown to inhibit growth of a pathogen in vitro, there is still only weak evidence that the required inhibitory concentration is present at the site in vivo. Thus, whether phytoalexins inhibit the growth of pathogens in vivo is not yet certain. The fact, however, that phytoalexin production accompanies the hypersensitive response and is stimulated by plant pathogens in both non-host species and in genetically resistant lines of host species suggests phytoalexins are important in vivo.

Control of Phytoalexin Levels

Although many phytoalexins have been isolated, identified and characterized with regard to their anti-microbial activites very little is known about the mechanism of their induction in host tissues. Production of many phytoalexins can, however, be elicited in response to chemical treatments or wounding, in addition to microbial infection (28, 58). Hargreaves and Bailey (42) have detected substances released from damaged bean cells capable of inducing production of phaseollin and phaseollidin in neighboring living cells. They have suggested that normally the elicitor is compartmentalized within live cells and released upon a hypersensitive interaction between the pathogen and these cells. They suggest that the elicitor diffuses to surrounding cells which produce and excrete the phytoalexins allowing the dead cells to accumulate them.

Other investigators have partially purified phytoalexin elicitors from incompatible races of various fungal pathogens (85,2,27,75). These, however, do not exhibit race specificity: i.e., they elicit similar amounts of phytoalexin in both resistant and susceptible hosts. It has recently been reported that race specificity of P. infestans is due to glucans from mycelia of P. infestans which suppress host phytoalexin production. Suppression activity is greatest with glucans from a compatible race (30,31). Wade and Albersheim (85) have reported, however, that race specificity of P. megasperma var. sojae is due to glycoproteins from this organism and that, although they do not elicit phytoalexins, can protect soybeans from compatible races. Glycoproteins from incompatible races provide more protection than those from compat-

ible races.

Concluding Remarks

Bacterial blight of cotton is a destructive disease caused by the bacterial pathogen, X. malvacearum. At present, there are many cotton varieties which show differential resistance to various races of the pathogen; one line, Im 216, is resistant to all 18 races. As with other incompatible host-pathogen relationships, growth of X. malvacearum in Im 216 cotyledons is inhibited soon after inoculation. The mechanism of inhibition is the subject of this work. More specifically, this work describes the partial purification of a phytoalexin produced in Im 216 cotton cotyledons in response to inoculation with X. malvacearum, R4.

CHAPTER III

MATERIALS AND METHODS

Solvents and Chemicals

Chloroform, hexane, ethyl acetate, methylene chloride, cyclohexane, toluene and methanol were either "glass distilled" grade from Burdick and Jackson Laboratories, Inc., 1953 S. Harvey St., Muskegon, MI 49422 or were "HPLC Reagent" or "Photrex" grade from J. T. Baker Chemical Co., Phillpsburg, NJ 08865. Water was glass distilled. Acetic acid and calcium carbonate were reagent grade from Fisher Scientific Co., Pittsburgh, PA 15219. Catechin was purchased from Sigma Chemical Co. Unless otherwise stated, all methanol solutions were aqueous. Volumes of water and methanol were measured in separate graduated cylinders and were added together.

Cotton Lines

Data indicate that Im 216 contains the resistance genes B_2, B_3 and b_7 as well as the polygene complex, B_{Sm}, and is immune to bacterial blight; i.e., exhibits no macroscopic symptoms under field levels of inoculum of any race of X. malvacearum (21,10). Acid delinted seeds were planted in a commercial mixture of peat moss and vermiculite (Jiffy Mix Plus, Jiffy Products of America, 250 Town Road, West Chicago, IL 60185) in 120 cm x 32 cm x 20 cm troughs. Plants were grown in

a greenhouse that was maintained at 32 ^{o}C during the day and 21 ^{o}C during the night. Cotyledons were inoculated 3 weeks after planting.

Bacteria

Race 3 (R3) and race 4 (R4) of X. malvacearum were used. Each race was supplied by Dr. William Johnson (Oklahoma State University, Plant Pathology, Stillwater, OK 74078). Both races are avirulent in Im 216 (2).

Bacterial strains were maintained on potato-carrot-dextrose agar slants as described by Essenberg et al. (32) except that the yeast extract was omitted. Inoculum cultures were started by inoculating a 0.8% solution of Difco nutrient broth (Difco Laboratories, Detroit, MI) from an agar slant. The inoculated broth was incubated overnight (16 h) at 30^{o}C with shaking (200 rpm). An aliquot of broth was removed and pipetted into 200 ml of a sterile saturated solution of calcium carbonate to yield 1-5 x 10^{6} colony forming units (cfu)/ml. The extract bacterial concentration was determined by the dilution plating technique using Difco nutrient agar.

Inoculation

Cotyledons were inoculated 3 weeks after planting in the following manner. Using a 22 gauge syringe needle, one scratch, 1-2 mm in length, was made on the lower epidermis of cotyledons in each of four panels. A 10 cc syringe without a needle was used to infiltrate the intercellular space. The syringe was loaded with the inoculum which was prepared as described above, placed over each of the four wounds and the inoculum pushed into the intercellular space. An equal number of cotyledons

were inoculated in the same way with a sterile saturated solution of calcium carbonate as a control against inoculation damage.

Growth Curve

On days 0, 1, 2, 3, 4, 6 and 7 following inoculation (see above) 3 discs (0.9 cm in diameter) were removed from a cotyledon with a standard paper hole-punch. Total tissue removed was 1.0 cm^2. The discs were rinsed in 1 ml of a sterile calcium carbonate solution and ground with a sterile mortor and pestle containing 1 ml of the same calcium carbonate solution. The suspension was diluted and plated and the bacterial colonies were counted two days later. Bacterial populations are reported as colony forming units per cm^2 of cotyledon tissue.

Purification

All work was carried out under subdued light or under UV-deficient illumination from General Electric FO6T12/GO gold fluorescent lamps.

Extraction

Cotyledons from 150-200 plants were harvested 3 days after inoculation, weighed and rinsed in deionized water. Intercellular solutes were extracted with either 50% aqueous methanol or 40% aqueous methanol by the method of Klement (50). The washed cotyledons were cut into distal and proximal halves with a razor blade and the halves were placed into a 1000 ml filter flask containing 750 ml of the extraction solvent. The flask was sealed with a rubber stopper and the contents were subjected to a vacuum (26 mm) for 3 min and the vacuum was quickly released. The sudden increase in pressure forced the solvent through the

cut edges of the cotyledon halves into the intercellular space. To accomplish complete infiltration, the procedure was repeated except that the vacuum was held for only 30 s before release. The extraction solvent was then discarded and the cotyledon halves were stacked (20-30 halves per stack) and placed, cut edges down into 2.5 cm x 10 cm "Nalgene" centrifuge tubes, specially fitted with metal grids, and centrifuged at 1000 x g for 10 minutes. Methanol was evaporated from the intercellular fluid extracts (expressed solution) by a nitrogen stream at 30 ^{o}C. The extract from Im 216 cotyledons which were inoculated with X. malvacearum was designated "IX"; the extract from calcium carbonate-inoculated cotyledons was designated "IC".

Thin Layer Chromatography

Following removal of methanol from the IX and IC extracts, the remaining aqueous solutions were extracted with three volumes of ethyl acetate, each volume equal to that of the original extract. The ethyl acetate extracts were rotary evaporated to dryness at 30^{o}C. Each residue was redissolved in 0.40 - 0.60 ml of methanol and each was streaked onto a 20 cm x 20 cm silica gel G plate, 250 µm thick (Analtech, Inc., 75 Blue Hen Drive, Newark, DE 19711) which had been prerun in methanol and activated by drying for 30 min at 60^{o}C in vacuo. The chromatograms were developed with a mixture of hexane-ethyl acetate-methanol (60:40:1) --system I; or with chloroform-methanol (70:30)--system II. Silica gel was scraped from the plates with a clean razor blade. The adsorbed substances were eluted by washing the gel three times with methanol (5 ml/cm^{3} gel) and pouring the slurry through Whatman No. 4 (Whatman, Inc., Clifton, NJ 07014) filter paper to remove the silica gel.

SEP-PAK Chromatography

Following removal of methanol, the IX and IC crude extracts were passed through a SEP-PAK C_{18} cartridge (Waters Assoc., Inc., Milford, MA 01757) which had been prepared by passing 2 ml of methanol followed by two 5 ml volumes of water through it. Sample components which remained on the SEP-PAK cartridge were eluted first with 5 ml of water, then successively with 5 ml volumes of varying percentages of aqueous methanol as described in the "RESULTS" chapter.

High Performance Liquid Chromatography

Following either thin layer or SEP-PAK chromatography, samples were subjected to high performance liquid chromatography (HPLC) on either a Waters 3.9 mm x 30 cm μBondapak CN column or on a Beckman 4.6 mm x 26 cm "ULTRASPHERE" ODS (C_{18}) column (Beckman Instruments, Inc., Irvine, CA 92713) in various aqueous methanol solutions. A 3.9 mm x 2.5 cm guard column packed with Whatman CO:PELL ODS, a C_{18} pellicular material, preceded the main column. Prior to and between each run the HPLC system was equilibrated with the running solvent by pumping 30-50 ml of the solvent through the columns at a flow rate of 1.5 ml/min. All samples were dissolved in the running solvent and filtered through a 0.45 μm Millipore filter (Millipore Corporation, Bedford, MA 01730) before injection onto the column. Sample volumes were 0.40 - 1.0 ml. Solvent gradients were of either step or continuous (60 ml total volume) type. Continuous gradients were generated by two cylindrical vessels connected in series. Thirty milliliters of water and a stirring bar were placed in the vessel from which solvent was removed while 30 ml of

70% or 100% methanol were placed in the other. Column eluate was monitored for absorbance at 254 nm.

Bioassay for Inhibitory Activity

During various stages of purification extracts and chromatographic eluates were assayed for activity inhibitory to the growth of X. malvacearum R4. Since the amount of phytoalexin could not be directly measured, all weights were expressed as "gram equivalent (geq.)" of phytoalexin; i.e., the amount of phytoalexin present in cotyledons of a given gram weight. If samples contained only ethyl acetate or methanol, they were concentrated at 30°C with a stream of nitrogen to 0.20 - 0.40 ml and transferred to sterile 6 mm x 5 cm test tubes. If they contained only water, they were lyophilized, redissolved in 15 ml of methanol (lyophilization flask was rinsed with three 5 ml volumes), then concentrated and transferred to the sterile tubes. If the samples contained aqueous methanol, the methanol was evaporated at 30°C with a nitrogen stream and the remaining water was lyophilized and treated as above. After transfer to the sterile tubes, the methanol was evaporated to dryness at 30°C with a nitrogen stream. A culture of X. malvacearum R4 growing logarithmically in 0.8% solution of Difco nutrient broth was diluted to 2.5×10^3 cfu/ml in nutrient broth. Then 0.20 ml of this culture was added to each of the sterile test tubes containing the dried sample and also to duplicate tubes containing no sample as controls. The tubes were placed in a beaker containing a water-moistened paper towel and incubated at 30°C, with shaking (200 rpm) for 16-18 hours. Bacterial concentrations at the beginning and end of this incubation were determined by the dilution plating technique. Each dilu-

tion was plated in duplicate. The number of generations (n) that each culture had undergone was estimated from the equation:

$$n = \frac{\log (cfu/ml)_f - \log (cfu/ml)_i}{\log 2}$$

where $(cfu/ml)_i$ and $(cfu/ml)_f$ represent the bacterial concentrations at the beginning and end of the incubation, respectively. Inhibitory activity was assessed by calculating the n/n_c ratio where n is the number of generation the culture undergoes in the presence of sample and n_c is the number of generations it undergoes in the absence of sample (control). A ratio of one indicates no inhibition while a ratio of zero indicates bacteristasis (no growth). A ratio of less than zero indicates bactericidal activity; i.e., killing of the initial population.

CHAPTER IV

RESULTS

Introduction

Previously, Essenberg, et al. (33) isolated the phytoalexin, 2,7-dihydroxycadalene from Im 216 cotton cotyledons inoculated with X. malvacearum R3. Their purification procedure included extraction of the intercellular solutes with 95% aqueous ethanol followed by thin layer chromatography on silica gel in hexane-ethyl acetate-methanol (60:40:1). Dihydroxycadalene chromatographed with an R_f of 0.35 and appeared as a dark, ultraviolet (UV)-absorbing band. The R_f region between 0.00 and 0.18, however, was also quite inhibitory to the growth of X. malvacearum R3. Herein are described methods for the partial purification of phytoalexin present in the low R_f region of the above thin layer system. After screening a series of races of X. malvacearum, R4 was chosen because it elicited the greatest level of inhibitory activity from intercellular solutes of Im 216 cotyledons.

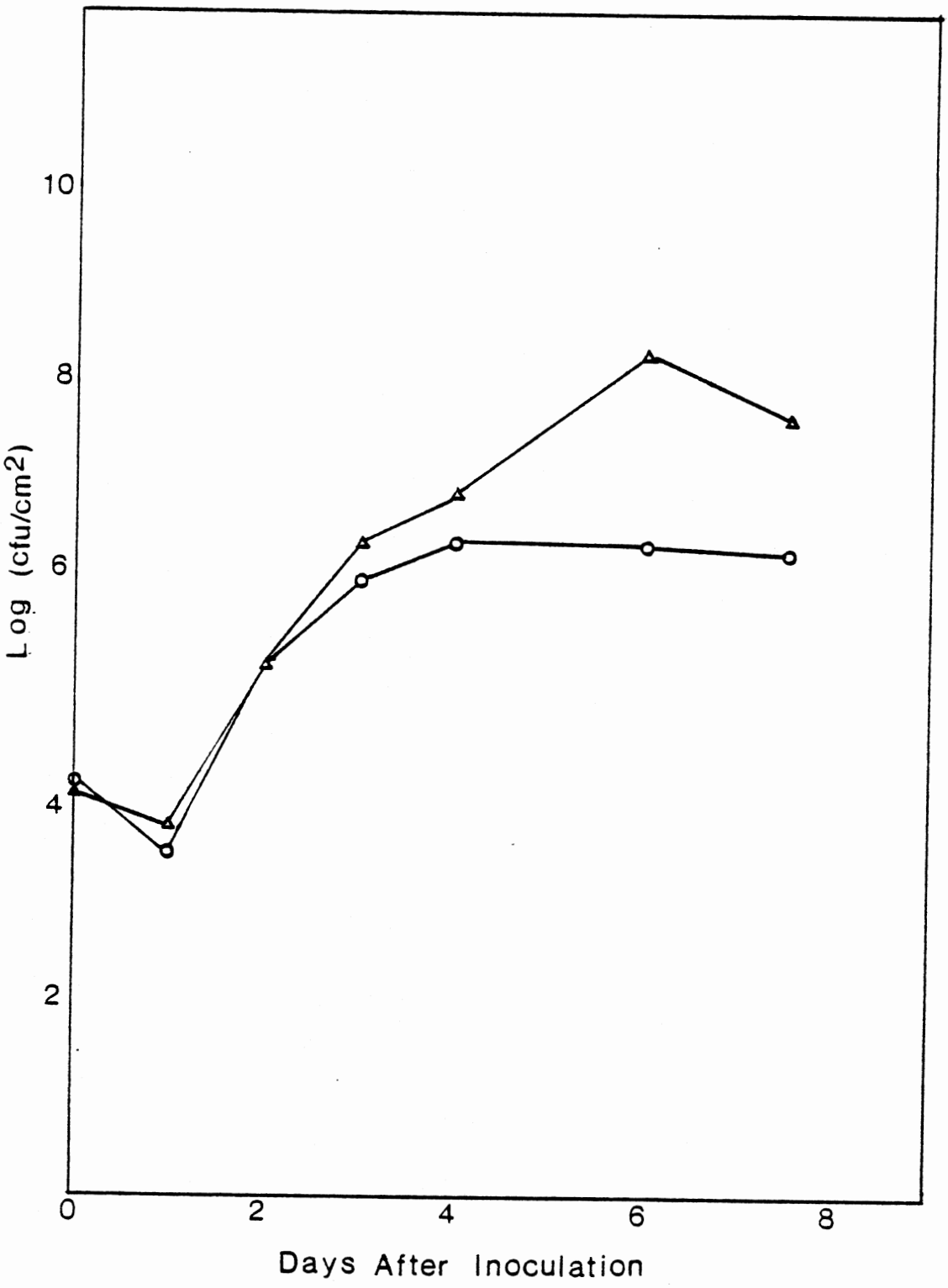
Growth of X. malvacearum R4 in Cotton Cotyledons

The growth patterns of X. malvacearum R4 in susceptible (Ac 44) and resistant (Im 216) cotton cotyledons were established by monitoring the growth of X. malvacearum R4 in 3-week old cotyledons of these cultivars for 7 days as described in "MATERIALS AND METHODS". The

growth curves shown in Figure 1 indicate that growth of R4 in Im 216 cotyledons was inhibited 3 days after inoculation whereas R4 continued to grow in Ac 44 to a maximum that was almost 100-fold greater than that in Im 216. For this reason, it was assumed that if Im 216 cotyledons did produce a phytoalexin in response to inoculation with R4, and if this phytoalexin was responsible for inhibition, it must be present in inhibitory levels 3 days after inoculation. A second assumption was that any phytoalexin produced must be secreted into the intercellular space where the bacteria reside for it to inhibit their growth. Therefore, we searched for phytoalexin by extracting solutes from the intercellular space 3 days after inoculation with R4.

Inoculation

Cotyledons from 150-200 Im 216 plants were inoculated 3 weeks after planting with either 1-5 x 10^6 cfu/ml of X. malvacearum R4 in a saturated solution of calcium carbonate or with a sterile saturated solution of calcium carbonate and harvested 72 h later as described in "MATERIALS AND METHODS". Cotyledons that were inoculated with R4 always showed reddening along the veins at harvest time. The degree of reddening depended on the actual inoculum concentration: cotyledons inoculated with 1-2 x 10^6 cfu/ml exhibited very little reddening whereas those inoculated with 3-5 x 10^6 cfu/ml showed more intense reddening. The reddening was presumed to be due to accumulation of anthocyanins. Inoculum levels above 5 x 10^6 cfu/ml produced browning and desiccation of entire cotyledons. Since intercellular solutes could not be efficiently extracted from desiccated tissue the inoculum level was kept at 1-5 x 10^6 cfu/ml.


10
8
6
4
2
0
2
4
6
8
Log (cfu/cm2)
Days After Inoculation

Cotyledons inoculated with a sterile saturated solution of calcium carbonate showed no reddening or desiccation. A few cotyledons, however, periodically exhibited small, brown rings at the inoculation sites where cells had probably been damaged by pressure of the inoculating syringe. This indicated that the reddening, severe browning and dessication were due to the interaction between X. malvacearum R4 and Im 216 cotyledon cells rather than to the stress of inoculation.

Extraction

Since the compounds to be purified moved more slowly on silica gel developed in system I (see "MATERIALS AND METHODS") than did 2,7-dihydroxycadalene, they were more polar than 2,7-dihydroxycadalene. To extract these compounds efficiently, a solvent more polar than 95% aqueous ethanol was chosen: 50% aqueous methanol.

After the inoculated cotyledons were weighed and washed, their intercellular solutes were extracted with 50% aqueous methanol as described in "MATERIALS AND METHODS". Typical results are shown in Table IV. The fresh weight of IX cotyledons was always less than that of the IC cotyledons. In addition, the average volume of intercellular extract from IX cotyledons was less than that from IC cotyledons. Whereas IC extracts always had a milky pale yellow appearance, IX extracts were a more intense yellow.

Thin Layer Chromatography With System I

Crude aqueous methanol extracts were subjected to thin layer chromatography as described in "MATERIALS AND METHODS". After development in system I, thin layer plates were examined under long ultra-

TABLE IV

EXTRACTION RESULTS USING 50% OR 40% AQUEOUS METHANOL

Cotyledon Type[1]	Number of Cotyledons[2]	Weight (g)[2]	Wt. (g)[2] / cot.	Extract Color
IX	126	33.5	0.26	Moderate-deep yellow
IC	110	49.4	0.45	Milky pale yellow

[1] See "ALPHABETICAL LISTING OF ABBREVIATIONS USED IN THE TEXT" (Pgs. xii-xiii).

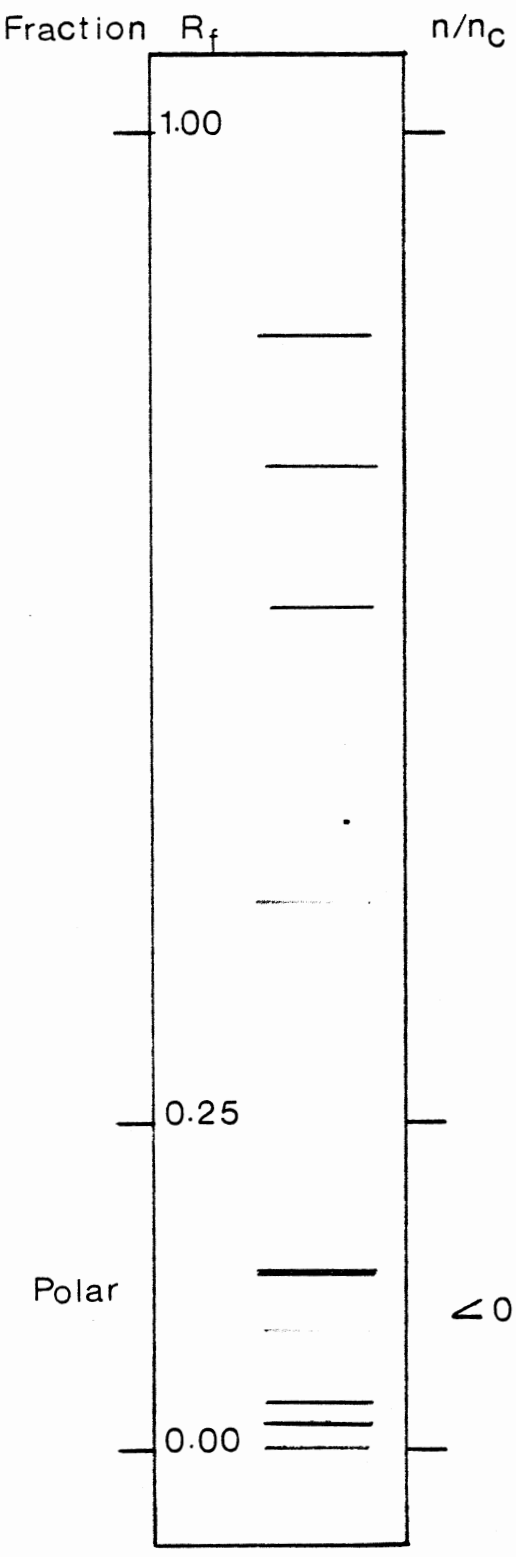
[2] These values represent averages from 9 preparations.

violet light. Figure 2 is a diagram of such a plate. The horizontal lines in the diagram represent the fluorescent bands seen under long UV light. The IX and IC extracts differed with respect to the intensity, but not the number of fluorescent bands observed. The brightest band from the IX extract was a sky blue one at R_f 0.17; the corresponding band at R_f 0.14 from the IC extract fluoresced much less intensely. In addition, a yellow band at R_f 0.09 and two slower moving blue bands at R_f 0.04 and 0.02 in the polar region from the IX extract exhibited brighter fluorescence than did corresponding bands from the IC extract. In contrast, there was no difference between extracts with respect to the fluorescence intensity of the less polar bands (R_f 0.42 - 0.83).

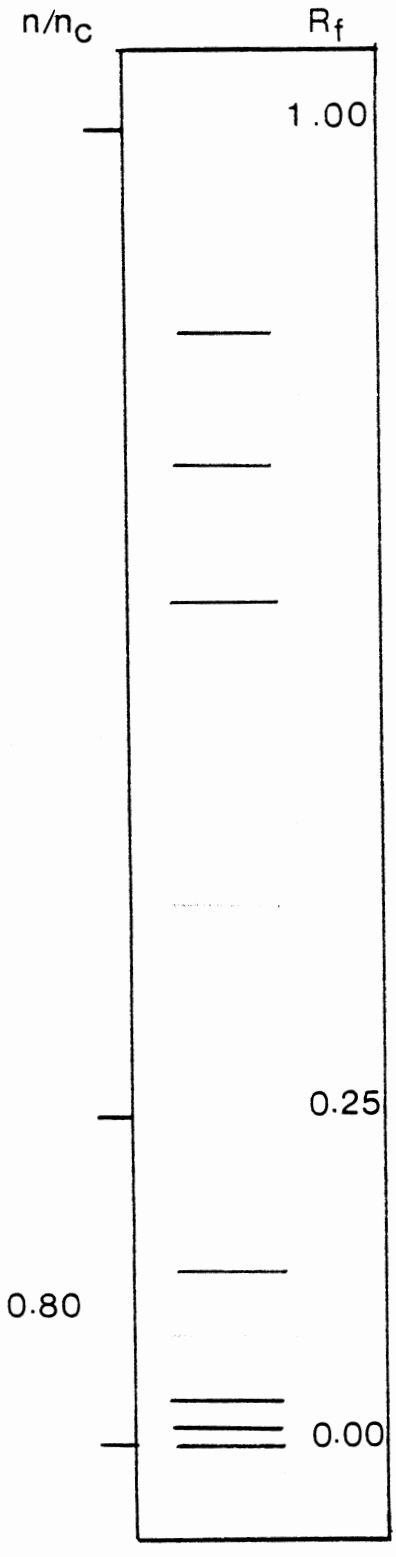
To establish if inhibitory compounds more polar than 2,7-dihychoxycadalene were extracted by the 50% aqueous methanol, adsorbed solutes from the polar region, R_f 0.00 - 0.25, were eluted from the gel and 1 geq of those solutes was bioassayed as described in "MATERIALS AND METHODS". The results are shown as "n/n_c" ratios (explained in "MATERIALS AND METHODS") in Figure 2. Whereas IX solutes exhibited bactericidal activity ($n/n_c < 0$), those from IC extract were only weakly inhibitory. An n/n_c ratio of 0.80 indicates X. malvacearum doubled 6.4 times instead of 8.0 (control) during the 16 hour incubation time. This difference was within the range of experimental error.

Development of the Second TLC Solvent System

The fact that extracts of IX cotyledons were more inhibitory than those from the inoculation control ("differential" inhibition) suggested that Im 216 cotyledons produced phytoalexin. To further resolve, by TLC, compounds found in the polar region (R_f 0.00 - 0.25) requires a


Fraction R_f
n/n_c
1.00
0.25
Polar
$\angle 0$
0.00

IX

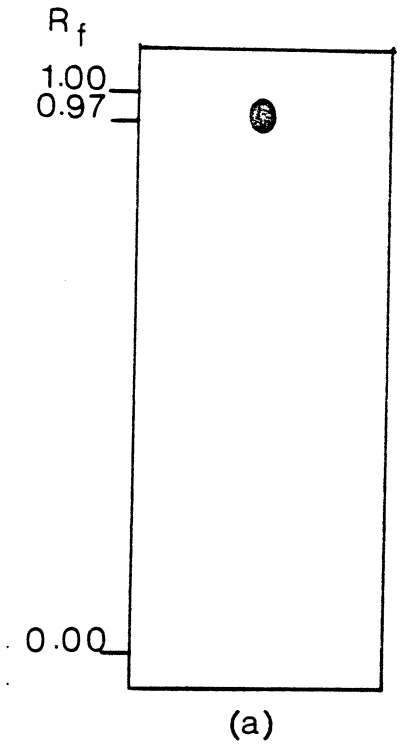

n/n_c
R_f
1.00
0.25
0.80
0.00

IC

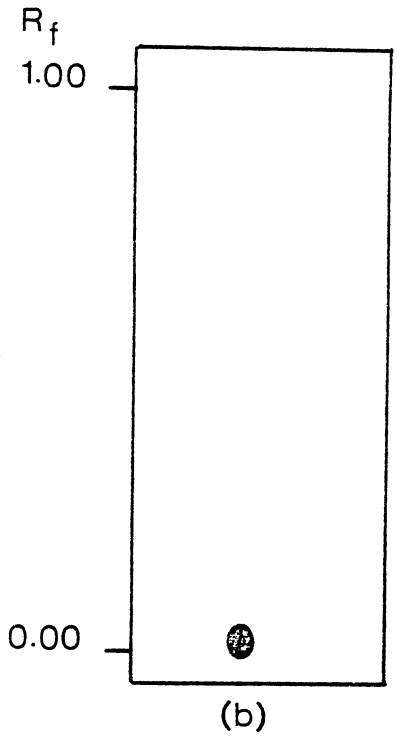
more polar solvent system than system I. The mobility of commercial catechin on silica gel plates containing a fluorescent indicator was used to evaluate other solvent systems (see "DISCUSSION" for explanation). Catechin was spotted onto 10 cm x 20 cm silica gel plates containing a fluorescent indicator and chromatographed in the various systems. Following development, plates were examined under short UV light for quenching of the fluorescent background.

The four solvent systems initially tried were: 2% aqueous acetic acid; chloroform-acetic acid (90:10); chloroform-methanol (90:10), and toluene-ethyl acetate (55:45). Representative diagrams of plates developed in these system are shown in Figure 3. The circles represent fluorescence quenching due to catechin. In 2% aqueous acetic acid, catechin migrated just behind the solvent front, R_f 0.97, while in chloroform-acetic acid (90:10) catechin remained at the origin. Neither of these systems would resolve compounds with polarities similar to that of catechin. Toluene-ethyl acetate (55:45) and chloroform-methanol (90:10) moved catechin only short distances; R_f 0.12 and R_f 0.20, respectively. In addition, trailing was observed in the aromatic solvent system. These results suggested that a chloroform-methanol mixture with greater than 10% methanol might move catechin to an R_f between 0.20 and 0.97.

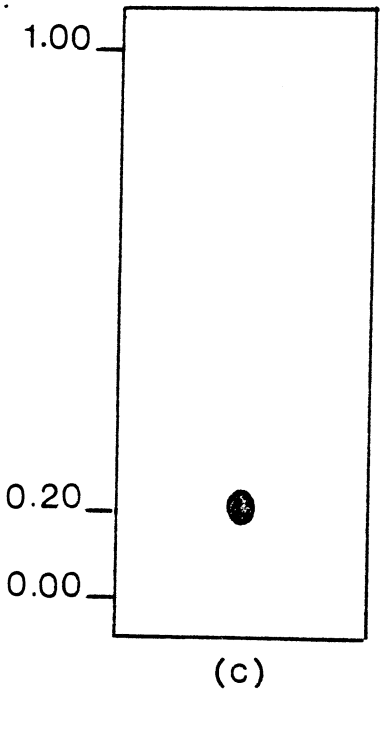
Two other chloroform-methanol ratios were tried: 80:20 and 70:30. The results are shown in Figure 4. As predicted, catechin moved further in each of these chloroform-methanol mixtures than it did in the 90:10 mixutre. Chloroform-methanol (70:30) was chosen for the second solvent system since up to 70% of the chromatogram was available for resolving compounds more polar than catechin and 30% for resolving compounds less



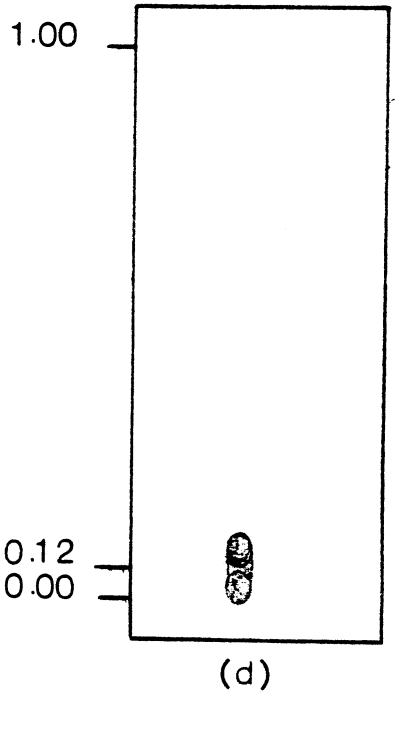
(a)



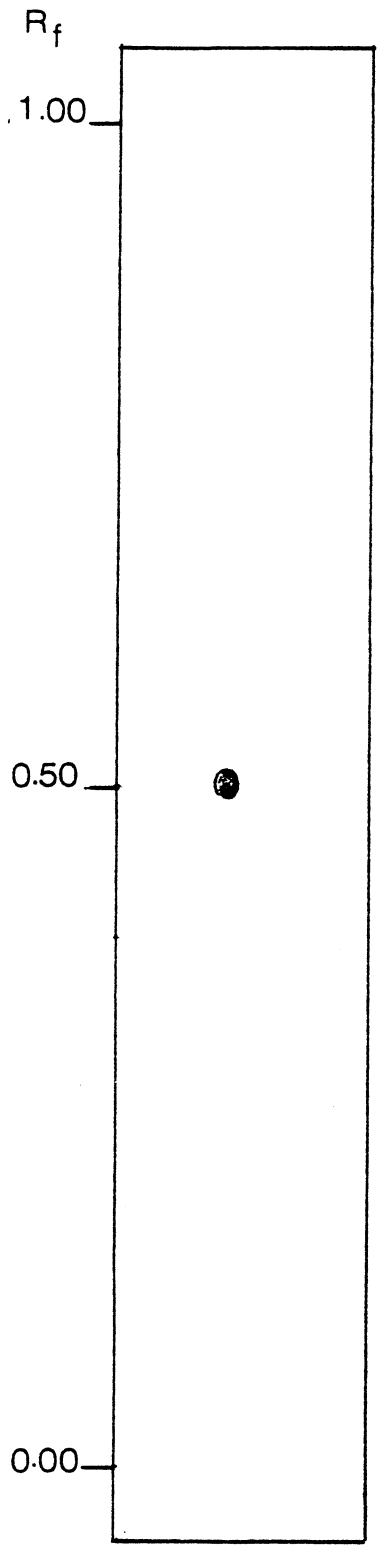
(b)



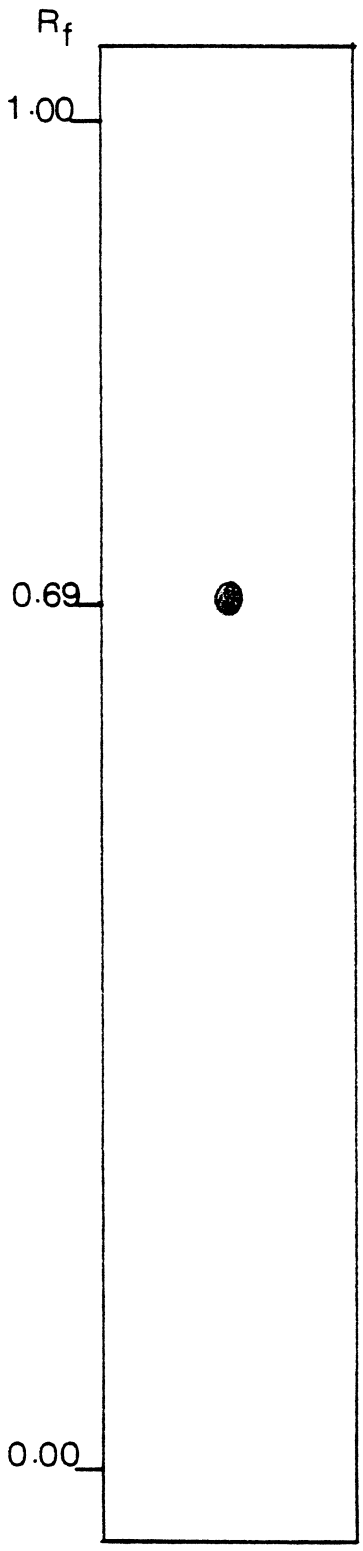
(c)



(d)



(a)



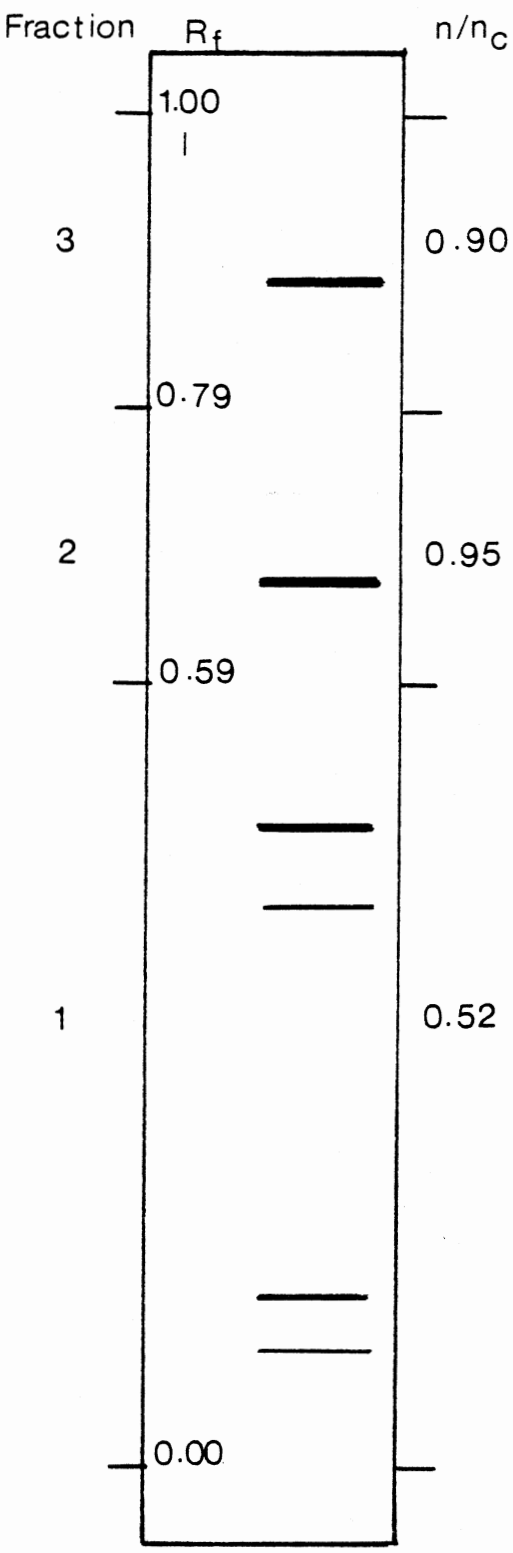
(b)

polar than catechin.

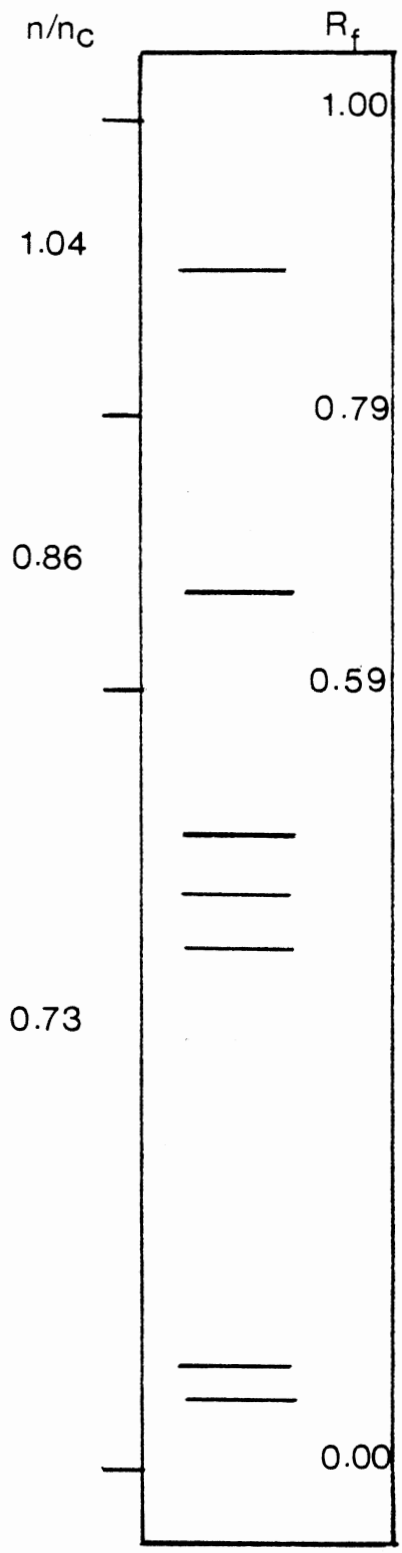
After chromatographing the crude intercellular fluid extract in system I, the region R_f 0.00 - 0.25 was scraped and the adsorbed solutes eluted. The eluate was concentrated and chromatographed in system II; results are shown in Figure 5. The four fluorescent bands seen on the first plate were resolved into eight fluorescent bands. In addition, there was no way of knowing how many non-fluorescing compounds were present. As with the first solvent system, the intensity of some of the bands was the only apparent difference between extracts.

To find the position of the polar inhibitor(s), the plate was divided into 3 fractions (R_f 0.00 - 0.59; 0.59 - 0.79; 0.79 - 1.00) and the solutes were eluted and 1 geq was bioassayed as with the first TLC system. The results are shown in Figure 5. Differential inhibitory activity was associated with fraction 1; i.e., the greatest amount of inhibition was caused by fraction 1 solutes from the IX extract (IX1). Fractions 2 and 3 of either extract did not inhibit the growth of X. malvacearum, R4. Thus, the compounds responsible for the bright blue fluorescent bands in fractions IX2 and IX3 may not be important in the inhibition of X. malvacearum inside the cotton cotyledons.

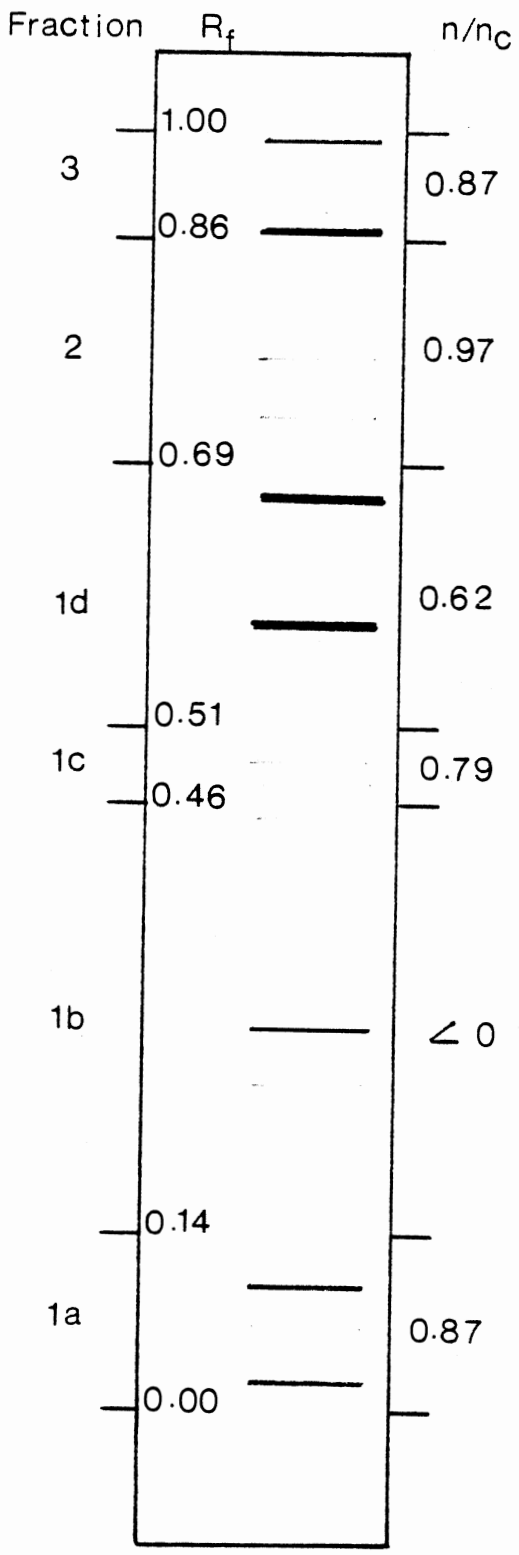
In order to find out where in fraction 1 inhibitor activity was, this fraction was subdivided into 4 fractions and the adsorbed solutes were eluted and 3 geq were bioassayed as before. The results are shown in Figure 6. Solutes from fractions 1a, 1c and 1d of both extracts exhibited weak to moderate inhibition, while those from IX1b and IC1b were bactericidal and strongly inhibitory, respectively. Thus, the pathogen could grow, although slowly, in the presence of IC1b solutes, but could not overcome the toxicity of the IX1b solutes. This was


Fraction
R_f
n/n_c
1.00
3
0.90
0.79
2
0.95
0.59
1
0.52
0.00

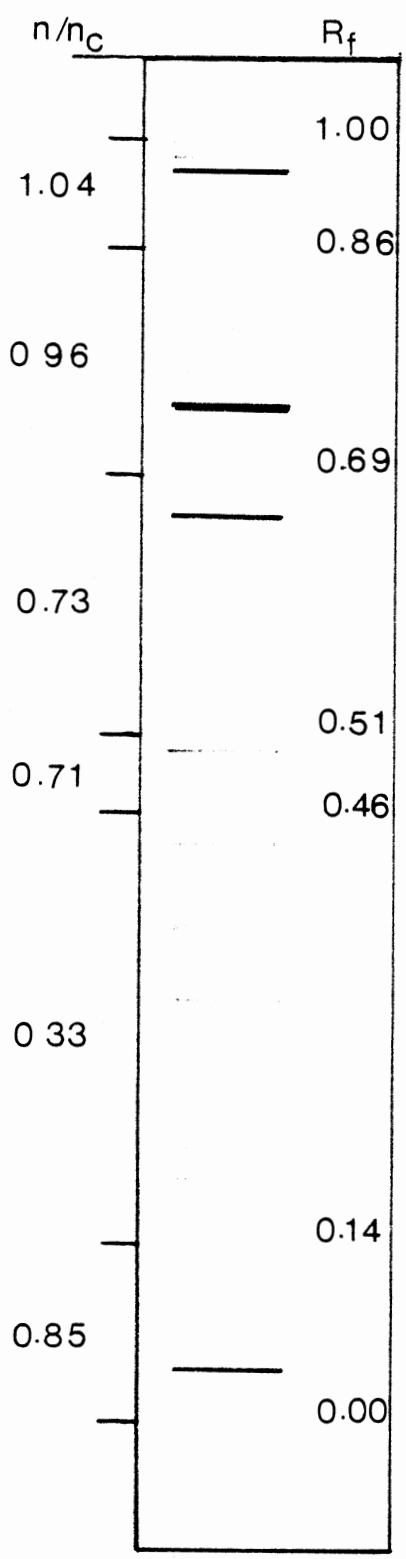
IX


n/n_c
R_f
1.00
1.04
0.79
0.86
0.59
0.73
0.00

IC


Fraction
R_f
n/n_c
1.00
3
0.87
0.86
2
0.97
0.69
1d
0.62
0.51
1c
0.79
0.46
1b
< 0
0.14
1a
0.87
0.00

IX


n/n_c
R_f
1.00
1.04
0.86
0 96
0.69
0.73
0.51
0.71
0.46
0 33
0.14
0.85
0.00

IC

interpreted as being due to the presence of one or more phytoalexins in the IX extract which were either not present in the IC extract or were present in lower levels.

If the inhibition exhibited by solutes of IClb was due to compounds other than phytoalexins (constitutive inhibitors) and if they were also present in fraction IXlb, they needed to be resolved from any phytoalexin(s) also in IXlb. The possible sources of these constitutive inhibitors are discussed in the "DISCUSSION". One possibility for further purification of these compounds was a third thin layer chromatographic system. Since 2,7-dihydroxycadalene was easily oxidizable and therefore unstable on silica gel, however, it was possible the more polar phytoalexin was also unstable; therefore, its exposure to silica gel plates needed to be minimized. Dihydroxycadalene was purified using high performance liquid chromatography following TLC and, therefore, HPLC was used for further purification of the more polar phytoalexin. In addition, it was found that the first TLC (system I) could be omitted. Instead, the 50% aqueous methanol extract was chromatographed directly in system II before HPLC. This yielded the same results as when the polar fraction from TLC in system I was rechromatographed in system II.

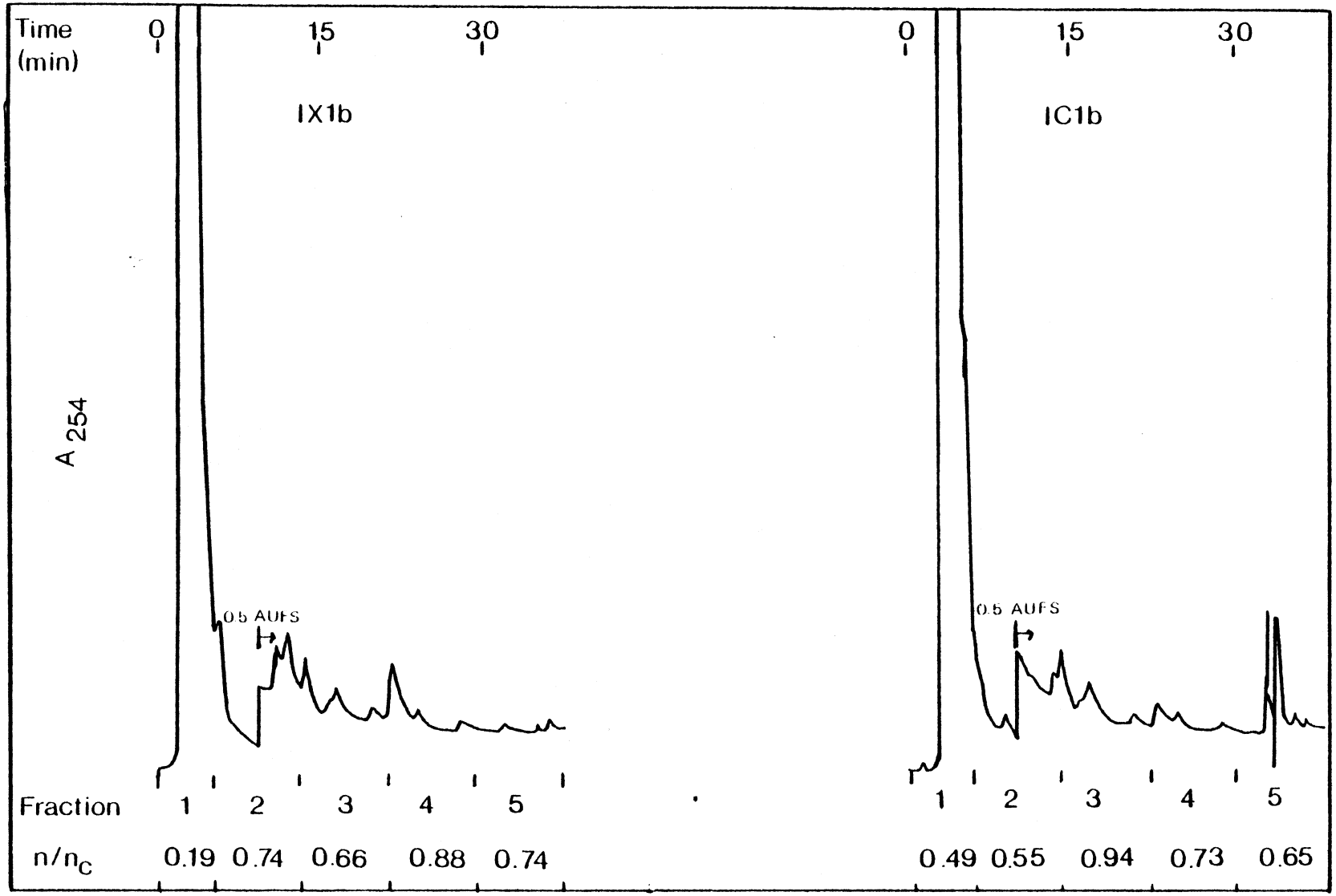
Development of HPLC Systems

There are two ways in which samples may be chromatographed on HPLC: normal phase and reverse phase. Normal phase is defined as having a stationary phase that is more polar than the mobile or running phase while reverse phase utilizes a stationary phase which is less polar than the mobile phase. In normal phase systems silica is typically

used as the stationary phase and organic solvents which are less polar than methanol are used as the mobile phase. Reverse phase systems typically use silica which has been derivatized with aliphatic hydrocarbons of varying carbon chain length (C_8 and C_{18} are the most common lengths). The hydrocarbons may contain functional groups such as cyano(-CN) or phenyl ($-C_6H_5$) to improve selectivity.

Because of its highly polar nature, a silica support (μPorasil) and methylene chloride-cyclohexane (90:10) system was tested first. Nearly all components of IXlb remained absorbed to the silica until the column was washed with a methylene chloride-methanol (50:50) mixture. Not only did this procedure not provide good resolution of IXlb components, but the use of hydroxylating solvents such as methanol drastically decreases the life of silica columns. For this reason, reverse phase systems were tested.

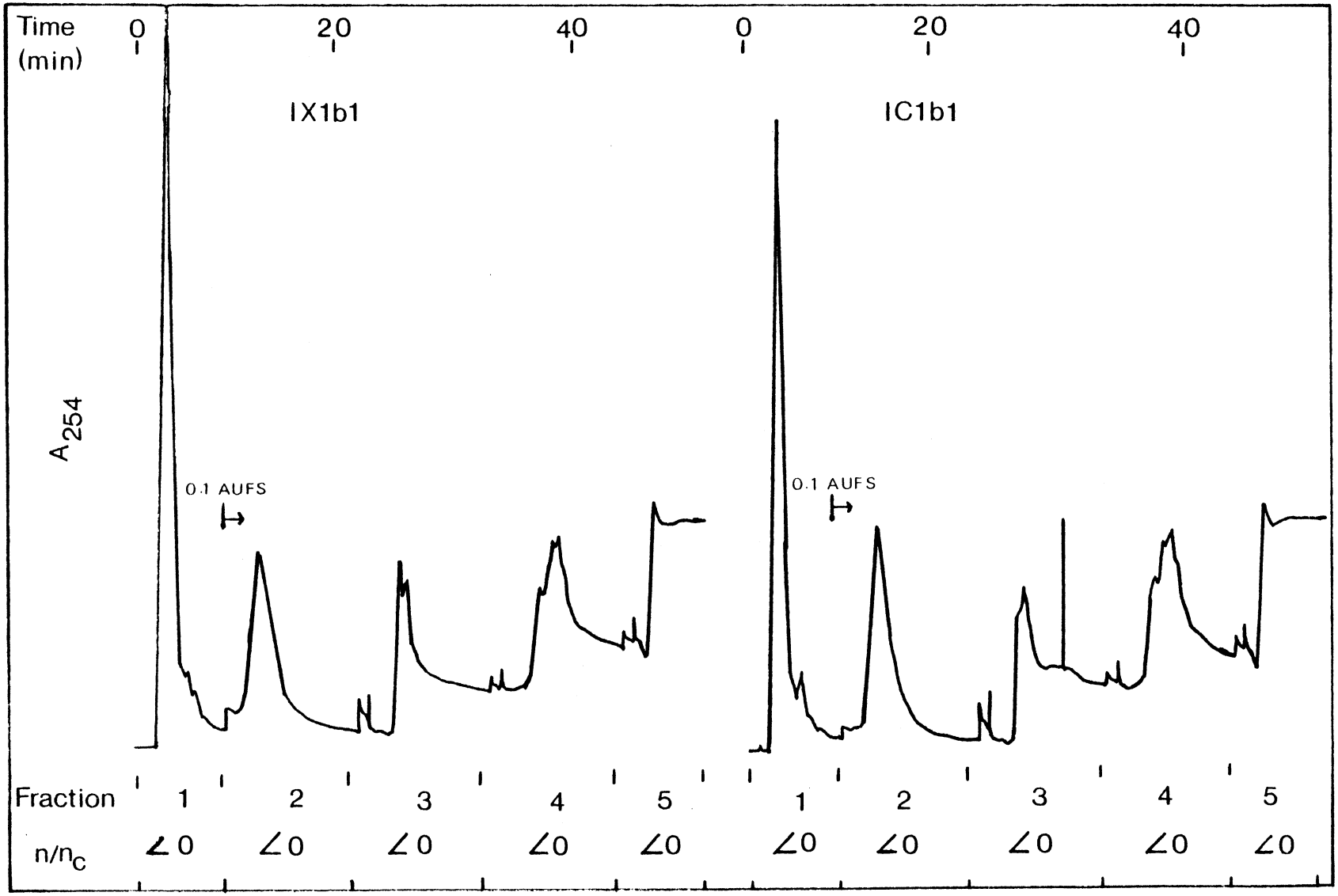
A column of intermediate polarity, μBondapak CN, was chosen. This material is silica derivatized with a saturated C_{18} hydrocarbon containing a cyano functional group which imparts the intermediate polarity to the non-polar C_{18} hydrocarbon. Aliquots of fraction 1b solutes from IX and IC extracts were dissolved in 40% aqueous methanol, filtered and injected onto the guard column connected to a μBondapak CN column as described in "MATERIALS AND METHODS". A 40% to 100% methanol step gradient was passed through the column and fractions were collected for bioassay. Figure 7 shows the elution profile obtained with this system. The IXlb and IClb profiles were very similar. With each sample, a large amount of material elutes without retention (Fraction 1b1). When bioassayed, fraction IXlb1 showed the


Time (min)
0
15
30
IX1b
A 254
0.5 AUFS
Fraction
1
2
3
4
5
n/n_c
0.19
0.74
0.66
0.88
0.74
0
15
30
IC1b
0.5 AUFS
1
2
3
4
5
0.49
0.55
0.94
0.73
0.65

greatest amount of inhibitory activity. The other fractions, including those from the control, exhibited weak inhibition. These results indicated fraction IX1b1 contained phytoalexin and, therefore, this fraction should be subjected to further HPLC.

The size and shape of the peaks in fractions IX1b1 and IC1b1 suggested that they contained many compounds. In order to resolve them, the IX1b1 and IC1b1 eluates were extracted with ethyl acetate, evaporated to dryness, redissolved in 20% aqueous methanol and subjected to HPLC as described in "MATERIALS AND METHODS" on μBondapak CN. The samples were chromatographed in 20% methanol for the first 20 minutes, followed by 40%, 80% and 100% methanol washes. The elution profile and bioassay results are shown in Figure 8. Each methanol solution eluted a different set of compounds.

The bioassay showed all fractions to be bactericidal. This suggested that all samples had been contaminated by something toxic which might have eluted from the HPLC column. Therefore, 20% aqueous methanol and 100% methanol column eluates were tested for toxicity; Table V shows the results. The 20% methanol eluate was much more toxic than was the 100% methanol eluate. Since 100% methanol is a better eluting solvent than 20% methanol and nothing toxic eluted in the 100% methanol, the column was probably not the source of the contaminant. A second possible source of contamination was examined: ethyl acetate, which was used to extract the HPLC eluates from water, after removing the methanol, in preparation for bioassay (see "MATERIALS AND METHODS"). The μBondapak CN column was eluted with 20% aqueous methanol, the methanol was removed and the eluate was divided into two equal volumes. One volume was extracted with ethyl acetate while the other volume was lyophili-


Time (min)
0
20
40
0
20
40
IX1b1
IC1b1
A_{254}
0.1 AUFS
0.1 AUFS
Fraction
1
2
3
4
5
1
2
3
4
5
n/n$_c$
∠0
∠0
∠0
∠0
∠0
∠0
∠0
∠0
∠0
∠0

zed and resuspended in methanol; each was bioassayed. Table V shows the results which indicate that ethyl acetate residues are toxic toward the growth of X. malvacearum R4, but that lyophilized 20% methanol column eluate is nontoxic. Future HPLC eluates were concentrated for bioassay by lyophilization. Fraction 1b1 from the 40%-100% methanol system was again chromatographed in a 20%-100% methanol step gradient on μBondapak CN and the eluates were bioassayed. The elution profiles were similar to those of Figure 8, however, the only fraction with any inhibitory activity was fraction 1 of IX1b1 which was bactericidal.

Development of SEP-PAK/HPLC Purification Procedure

In both HPLC systems described above phytoalexin chromatographed with very little retention. To minimize the accumulation of solvent impurities, a third HPLC system was avoided. Instead, cartridges filled with C_{18}-bonded silica (SEP-PAKS) replaced the previous thin layer chromatographic system. Also, a C_{18} column was used in place of the μBondapak CN column. In addition, 40% aqueous methanol was used as the extraction solvent instead of 50% in order to increase the selectivity of the extraction. The crude extract was subjected to SEP-PAK chromatography and washed successively with water, 30%, 60%, and 100% methanol as described in MATERIALS AND METHODS". The eluates were designated SP1, SP2, SP3 and SP4, respectively. All, but the 100% methanol fraction were chromatographed on a Beckman ODS (C_{18}) column in a continuous 0-100% methanol gradient as described in "MATERIALS AND METHODS" and the eluates were bioassayed. Figures 9-11 show the results.

TABLE V

EFFECTS OF COLUMN ELUATES AND ETHYL ACETATE RESIDUE ON GROWTH OF XANTHOMAS MALVACEARUM

Fraction	n/n_c
20% methanol eluate of μBondapak CN column[1]	0.42
100% methanol eluate of μBondapak CN column[2]	0.77
20% methanol eluates of μBondapak CN column, lyophilized[3]	1.00
20% methanol eluate of μBondapak CN column, extracted with ethyl acetate[4]	<0

[1]20 ml of 20% methanol were pumped through the μBondapak CN column at 1.0 ml/min and the methanol in the eluate was bubbled off with nitrogen. Remaining water was extracted 5 times with ethyl acetate which was dried for bioassay.

[2]Same as "1" except 100% methanol was used and no ethyl acetate extraction was necessary.

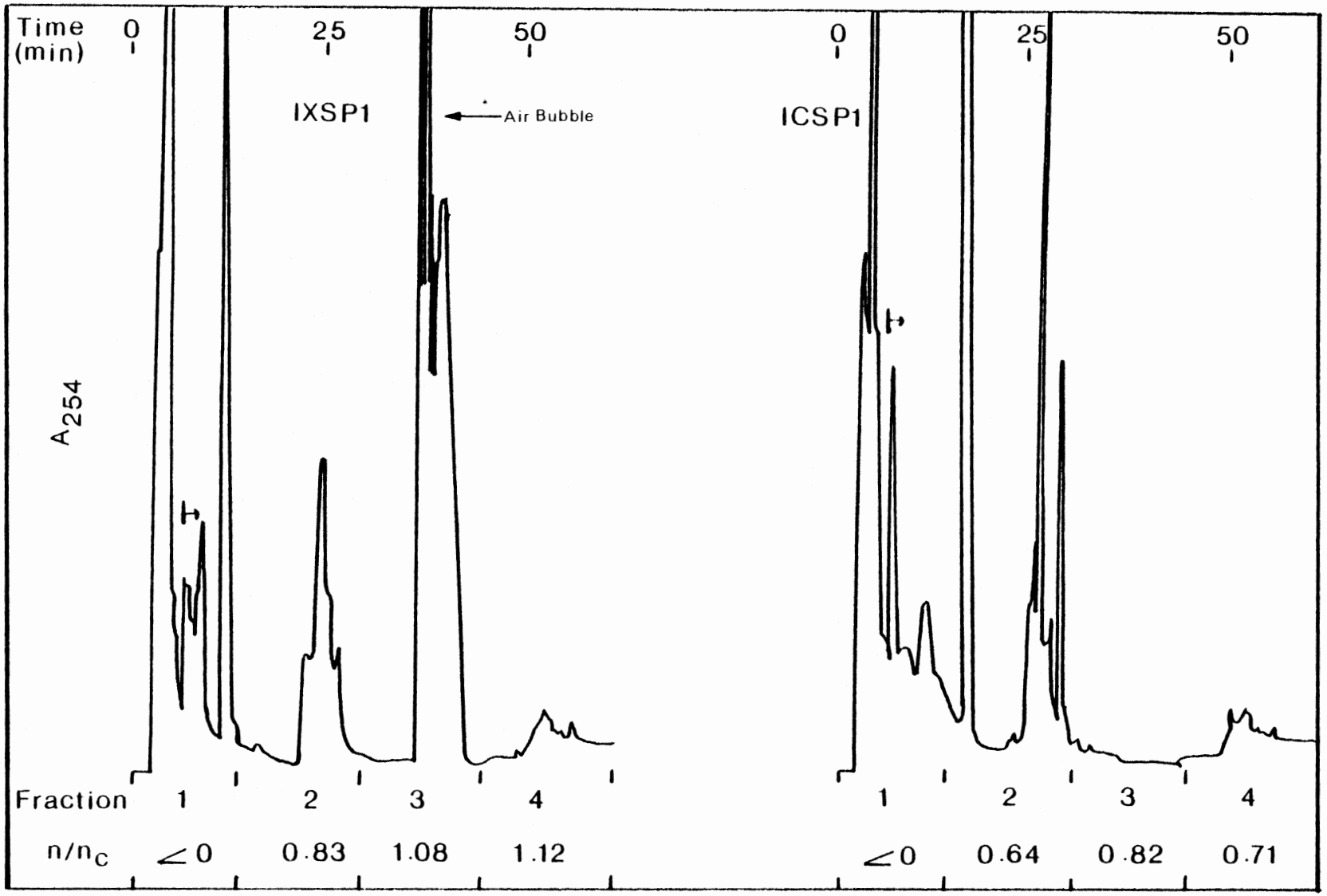
[3,4]Same as "1" except 40 ml of 20% methanol were eluted and 20 ml had the water lyophilized[3] and 20 ml had the water extracted with ethyl acetate[4].

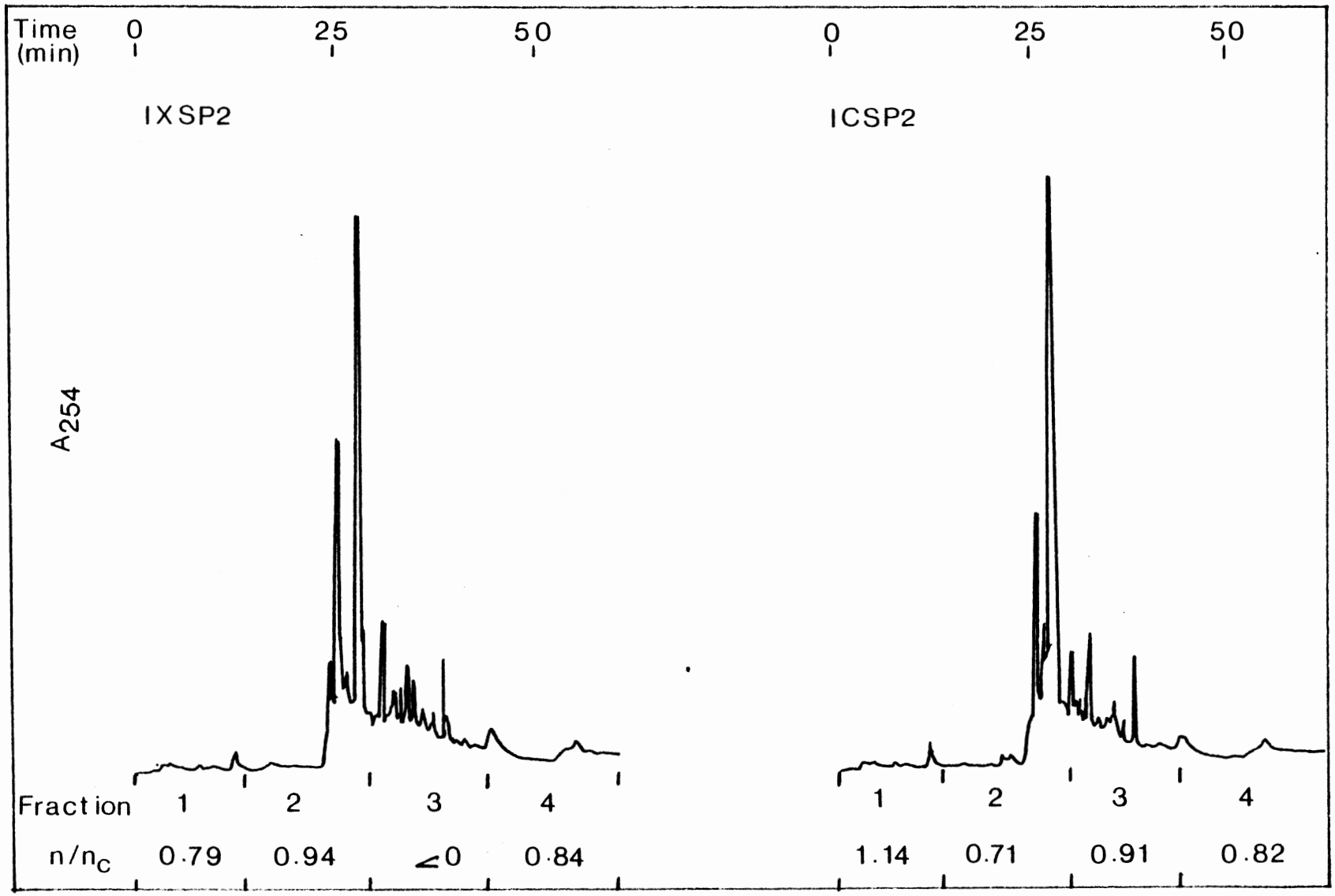
Figure 9 shows there was a large amount of highly polar material in SP1 eluates which partially chromatographed in the gradient. Due to the air bubble which had formed in the detector it was not known whether or not compounds had eluted in fraction 3 of IXSP1. Fraction 1 (SP1-1) from HPLC of both extracts contained highly toxic material while the remaining fractions exhibited little or no inhibitor activity.

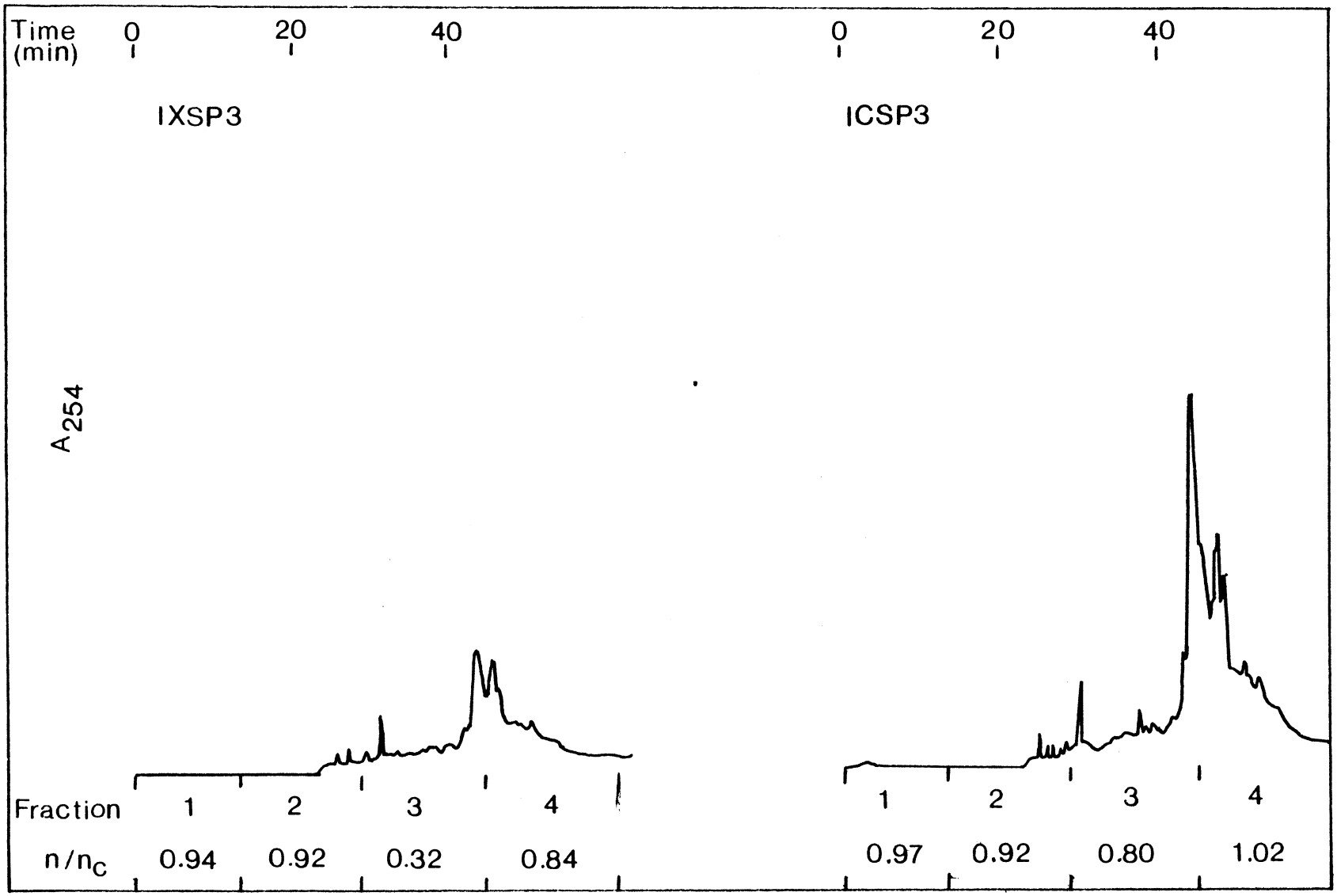
Figure 10 shows the HPLC results with the SP2 samples. The highly polar compounds which were present in the SP1 fraction were absent here; however, compounds from the latter part of fraction 2 of SP1 (SP1-2) may also have been present in SP2-2. Fraction 3 of SP2 (SP2-3) contained compounds exclusive to the 30% methanol eluate. As with the SP1 sample, very little eluted in the 100% methanol wash. Fraction SP2-3 exhibited strong differential inhibitory activity which was not observed in the other SP2 fractions. This suggested phytoalexin was in SP2-3.

The SP3 eluate (Figure 11) contained only a small amount of material not previously seen. The IC extract contained much more of this material (fraction 4). In addition, a few of the peaks seen in fractions 2 and 3 of the SP2 eluate were present here. The bioassay results supported the idea that phytoalexin eluted in fraction 3. Although not as strong as SP2-3, fraction SP3-3 from the IX extract exhibited differential inhibitory activity.

Since inhibitory activity was divided between fraction 3 of the SP2 and SP3 eluates, a new SEP-PAK fractionation scheme was tested in an attempt to obtain all differential inhibitory activity in a single SEP-PAK eluate. Crude extracts were prepared as before and subjected to SEP-PAK chromatography followed by successive washes with water (SP1'), 15% (SP2'), 35% (SP3') and 60% (SP4') aqueous methanol. The


Time (min)
0
25
50
IXSP1
Air Bubble
ICSP1
A_{254}
Fraction
1
2
3
4
n/n_C
< 0
0.83
1.08
1.12
< 0
0.64
0.82
0.71


Time (min)
0
25
50
0
25
50
IXSP2
ICSP2
A_{254}
Fraction
1
2
3
4
1
2
3
4
n/n$_c$
0.79
0.94
<0
0.84
1.14
0.71
0.91
0.82


Time (min)
0
20
40
0
20
40
IXSP3
ICSP3
A_{254}
Fraction
1
2
3
4
1
2
3
4
n/n_c
0.94
0.92
0.32
0.84
0.97
0.92
0.80
1.02

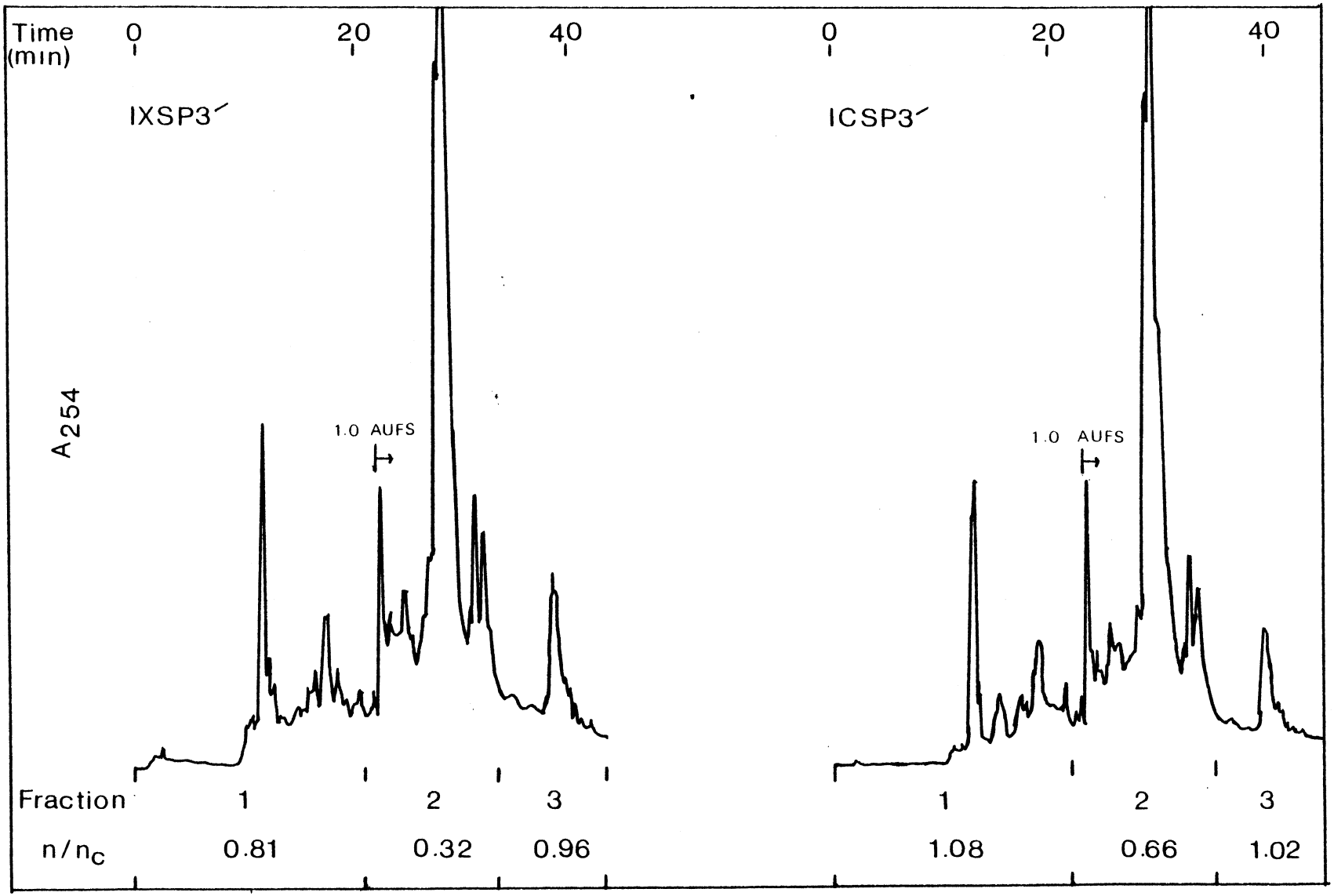
15% - 35% (SP3') and the 35% - 60% (SP4') eluates were chromatographed on the Beckman ODS column in a 0 - 70% aqueous methanol continuous gradient and the HPLC eluates were bioassayed.

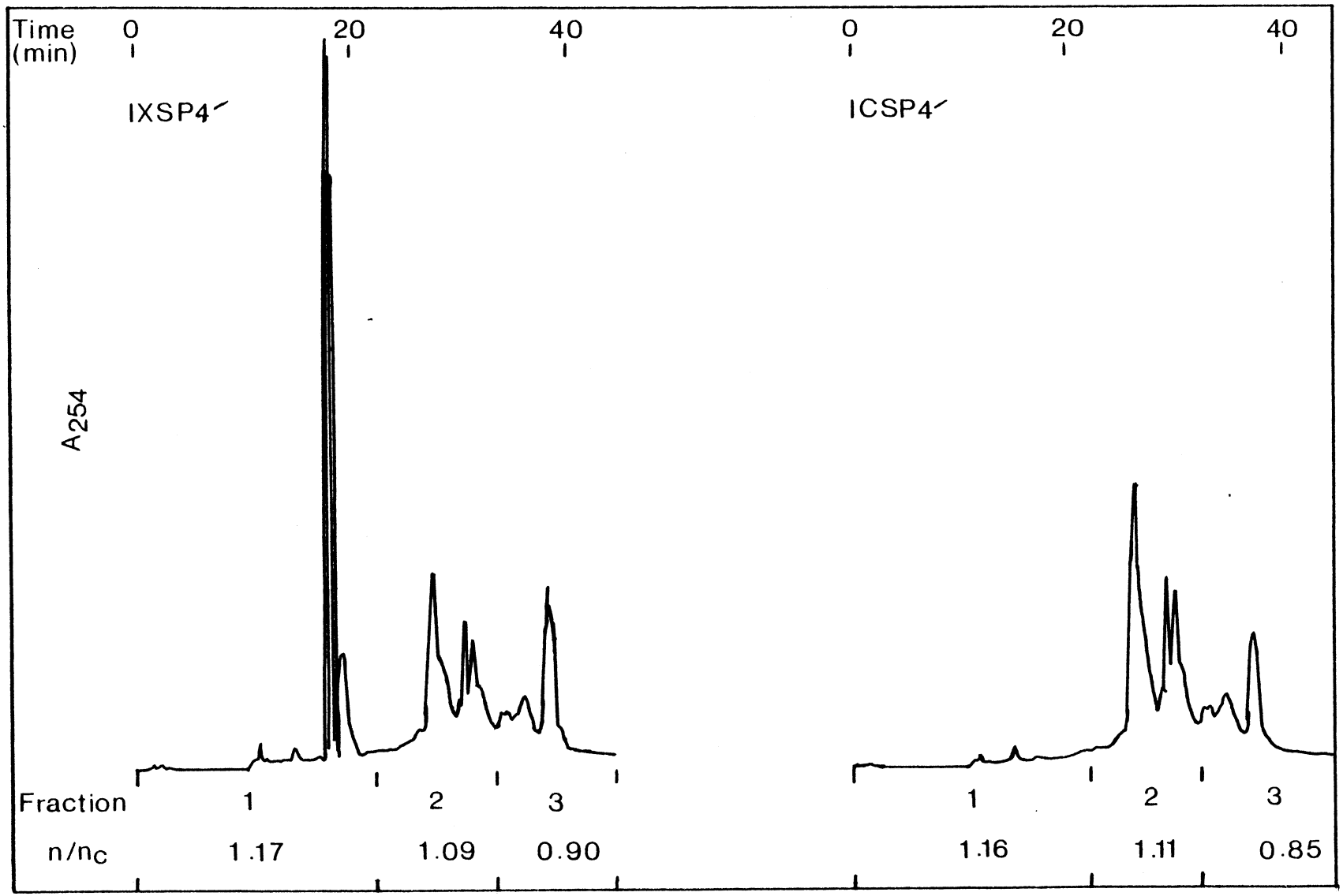
Figure 12 shows the results for SP3', the 15% - 35% methanol eluate. As in the 0% - 30% methanol eluate of the previous experiment (Figure 10), none of the highly polar material was present. However, two important differences were apparent: (1) the shallower gradient (0 - 70% instead of 0 - 100%) spread the peaks more and (2) peaks that were previously found in fractions 3 and 4 of the 30% - 60% SEP-PAK eluate now appeared in fraction 2 of the 15% - 35% SEP-PAK eluate. When bioassayed, differential inhibitory activity was observed in fraction 2.

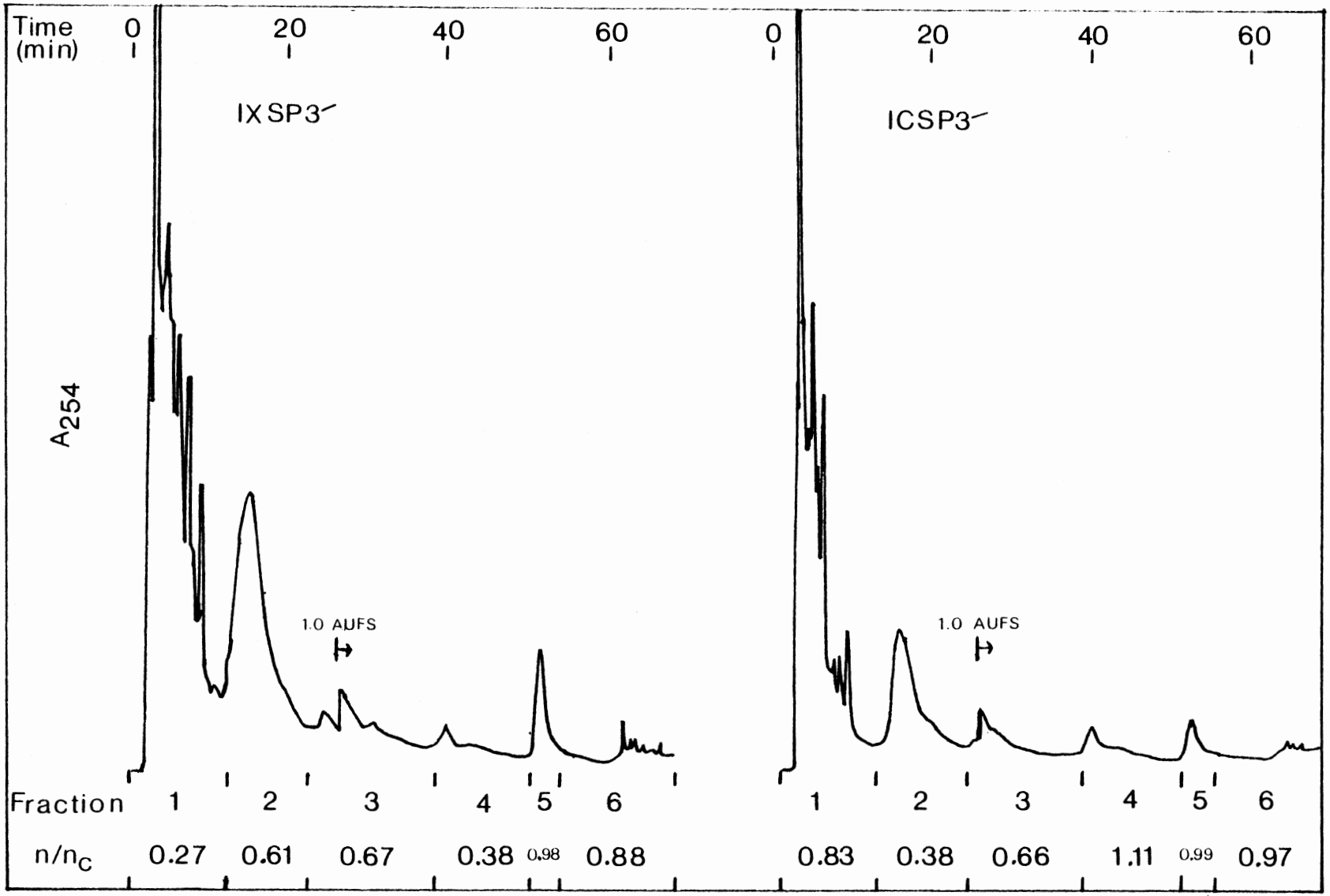
Figure 13 shows the HPLC profile for the SP4' eluate. Apparently, 35% methanol was not sufficient to elute all of the material responsible for the larger peaks in fraction 2 of SP3' since some of this material was also present in the 35% - 60% methanol (SP4') eluate. When bioassayed, however, none of the SP4' fractions were inhibitory. Therefore, the 15% - 35% methanol eluate from the IX extract contained phytoalexin.

In order to isolate pure phytoalexin from fraction 2 of IXSP3', the peaks needed to be resolved into their individual components. It was assumed the gradient was linear and, therefore, that fraction 2 eluted between 35% and 56% methanol. For this reason, a step gradient that began with 35% methanol was tested to resolve the peaks in IXSP3' and ICSP3' eluate. Figure 14 shows the results.

The 35% methanol eluted a large amount of material (fraction 1) from both extracts followed by a single peak (fraction 2) of moderate


Time (min)
0
20
40
IXSP3′
1.0 AUFS
A254
Fraction
1
2
3
n/nc
0.81
0.32
0.96
0
20
40
ICSP3′
1.0 AUFS
1
2
3
1.08
0.66
1.02

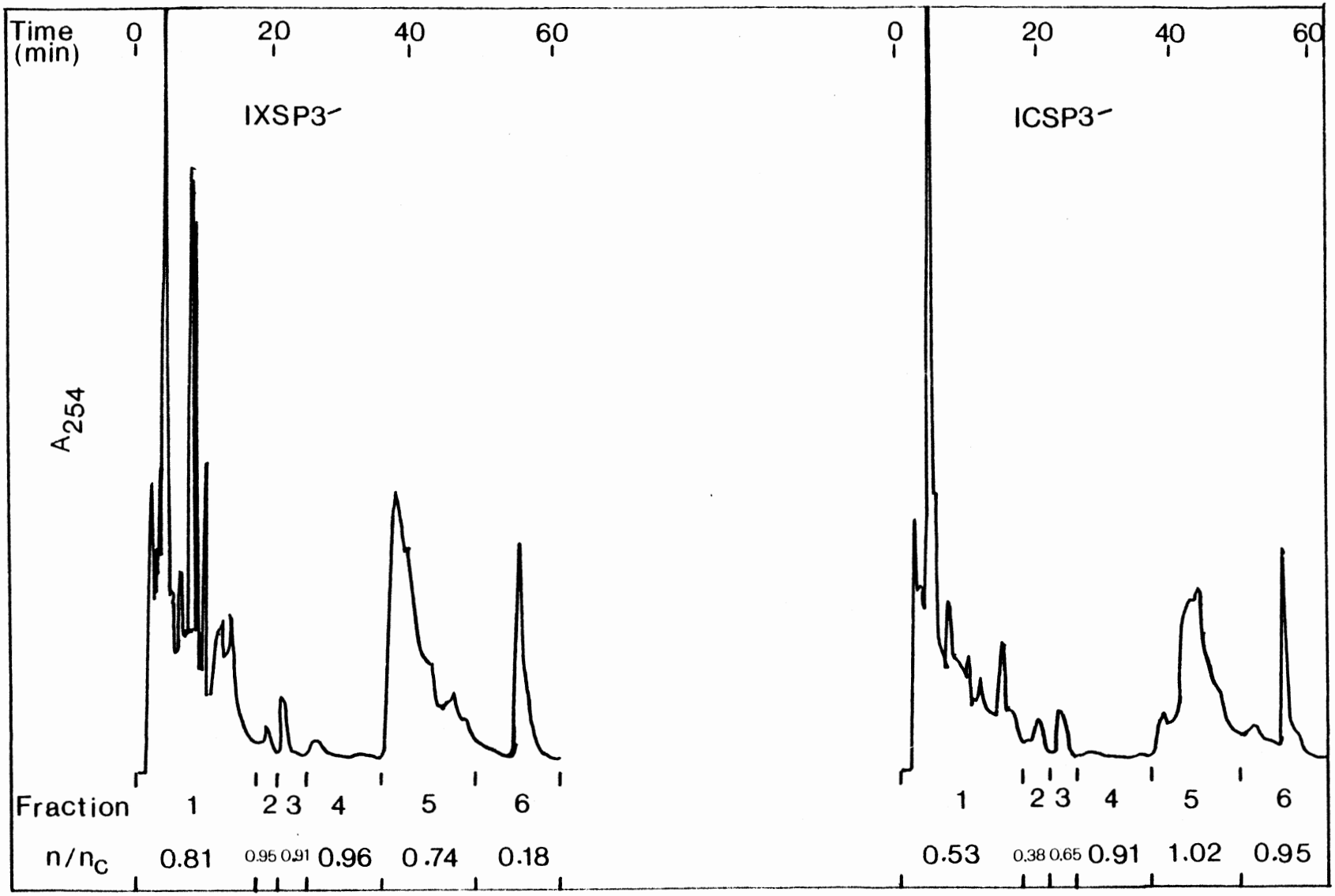

Time
(min)
0
20
40
0
20
40
IXSP4
ICSP4
A254
Fraction
1
2
3
1
2
3
n/nc
1.17
1.09
0.90
1.16
1.11
0.85


Time (min)
0
20
40
60
IXSP3⁻
ICSP3⁻
A254
1.0 AUFS
1.0 AUFS
Fraction
1
2
3
4
5
6
n/nC
0.27
0.61
0.67
0.38
0.98
0.88
0.83
0.38
0.66
1.11
0.99
0.97

intensity which was resolved from the multiple peaks in fraction 1.. The 50% methanol eluted a small peak (fraction 4) from each extract and the 67% methanol eluted a single peak (fraction 5). The small peaks seen in fraction 6 were eluted with 100% methanol. The peaks in fractions 2 and 5 of the IXSP3' had more than twice the area of the corresponding peaks from the ICSP3' eluate. In addition, the peak in fraction 5 was symmetrical which suggested it might be the result of a single compound.

When 6 geq were bioassayed, fractions 1 and 4 showed differential inhibitory activity. Since it was difficult to see any resemblance of these profiles to the profiles in fraction 2 of Figure 12, these bioassay results were not expected. Inhibitory activity in fraction 4 was hard to understand since there was low absorbance in that fraction. The differential inhibitory activity in IXSP3'-1 might have been due to the last eluted peak in that fraction since the corresponding peak in ICSP3'-1 was half the height of the IXSP3'-1 peak. For that reason, 12 geq of IXSP3' and ICSP3' were chromatographed in another step gradient that began with 25% methanol on the ULTRASPHERE C_{18} column. This was done in an effort to resolve the desired peak of fraction 1 from the other peaks. Results are shown in Figure 15.

The fraction 1 peaks were better resolved than with the previous HPLC system (Figure 14). The large peak in fraction 4 eluted with 50% methanol; this was not previously observed (Figure 14). When 6 geq were bioassayed, differential inhibitory activity was found in fraction 6, the 100% methanol wash. Since the 100% methanol wash was started after the 50% methanol, a methanol percentage between 50% and 100% might have eluted all or part of the peaks in fraction 6.


Time (min)
0
20
40
60
IXSP3⁻
ICSP3⁻
A_{254}
Fraction
1
2
3
4
5
6
n/n_c
0.81
0.95
0.91
0.96
0.74
0.18
0.53
0.38
0.65
0.91
1.02
0.95

CHAPTER V

DISCUSSION

Extraction

The general appearance of the cotyledons 3 days after inoculation depended on the inoculum level. In the inoculum range used, 1-5 x 10^6 cfu/ml, very little browning was observed; however, slight wilting occurred. Fresh weight of the IX cotyledons was usually less than that of the IC cotyledons (Table IV). In view of the slightly wilted appearance, the most likely cause of the decreased fresh weight was the loss of water which probably results from the hypersensitive interaction between Im 216 cells and X. malvacearum: host cell membranes were damaged which allowed electrolyte leakage and subsequent evaporation through stomatal openings. The events leading to the hypersensitive response are still unknown. It is known, however, that initiation of the hypersensitive response is irreversible within 20 min following inoculation (54).

The severity of the hypersensitive response in resistant hosts at a given inoculum level is related to the virulence level of the pathogen in susceptible hosts: the more virulent a pathogen is against a susceptible host, the more intense will be the hypersensitive response of the resistant host. In this way, resistant hosts might naturally select for the avirulent or less virulent races of a mixed population. Race 4 remained virulent on potato-dextrose-carrot agar during all, but

the last few preparations. All figures beyond and including Figure 15 represent preparations inoculated with R3.

Development of the Second TLC Solvent System

Catechin (see Figure 17) was used to evaluate new solvent systems because of the work of Venere (84). He (84) examined the possible roles of peroxidase and catechin in the resistance of Im 216 to X. malvacearum. Venere (84) found that within 2 h after inoculation with live X. malvacearum, total peroxidase activity began to increase in Im 216 cotyledons; no increase was observed in Ac 44 cotyledons. Shortly after peroxidase activity began to increase, growth of X. malvacearum in Im 216 cotyledons was inhibited and was followed by a rapid decline in the bacterial population. In contrast, X. malvacearum continued to grow in Ac 44 cotyledons where peroxidase activity remained constant. Venere (84) also reported that if catechin and hydrogen peroxide were mixed together in the presence of peroxidase that had been partially purified from the cell wall of Im 216 cotyledons, the resulting oxidation products of catechin were quite toxic to X. malvacearum.

The results of Venere (84) suggested the possibility that the inhibitory material in the IX polar fraction of system I (Figure 2) might be directly or indirectly related to the peroxidase catalyzed oxidation products of catechin. This idea was strengthened by the fact that catechin and its oxidation products remained at the origin when chromatographed in system I (results not shown). It was assumed that oxidation products of this compound would be more polar than catechin itself and, therefore, would chromatograph with lower R_f than catechin. For this reason, a chloroform-methanol mixture (70:30) was chosen that

moved catechin to an R_f value which would allow more polar oxidation products to be resolved (Figure 4).

Inhibition From IC Extracts

Throughout the purification, IC extracts have always exhibited some degree of inhibition. Sometimes chromatographic fractions of IC extracts were as inhibitory or slightly more inhibitory than corresponding fractions of IX extracts. For that reason, it was not assumed that the IX fraction with the greatest inhibitory activity necessarily contained phytoalexin. Instead, IX fractions with the greatest inhibitory activity relative to the activity of the corresponding IC fractions (differential inhibition) were assumed to contain phytoalexin and subjected to further purification. The inhibitory activity in IC extracts may have been due to one or more of the following: (1) intercellular solutes which became toxic as they were concentrated during extraction and bioassay; (2) intracellular solutes which are normally toxic, but do not come in contact with X. malvacearum in the intercellular space; or (3) low levels of phytoalexin which were elicited by the slight injury sustained by cotyledons during inoculation.

Concluding Remarks

The results presented here strongly support the hypothesis that X. malvacearum R4 or R3 elicits production of one or more polar phytoalexins in Im 216 cotyledons. The techniques employed have resulted in partial purification of the phytoalexin(s). Total purification might be accomplished through further HPLC which is based on solute characteristics other than polarity. Two such characteristics are

charge and molecular size. If the phytoalexin(s) has (have) acidic or basic functional groups, use of an ion pair reagent or variation of the pH of the running solvent, perhaps with a pH gradient, might achieve resolution from neutral compounds or from compounds of different pKa's. If total resolution can not be achieved with a C_{18} column, an ion exchange column might be more compatible with the variable pH system.

Molecular sieve chromatography may be useful if the phytoalexin(s) is (are) significantly different in size from the contaminating components. To find what type of HPLC molecular sieve column would be most useful, pilot runs could be made on Sephadex gels of various size (G-25, G-50, etc.). Eluates could be monitored for absorbance at 254 nm as with HPLC. Although it is possible that a Sephadex system sufficient to yield apparently pure phytoalexin might be found, HPLC columns offer a greater number of theoretical plates and may, therefore, achieve better separation of components close to each other in size.

A third method of improving phytoalexin purification might be to refine the extraction technique already employed prior to SEP-PAK chromatography. A search for a more selective extraction solvent could eliminate many of the components now extracted. If an aqueous methanol mixture were still used for extracting solutes from the intercellular space, it is possible that solutes could be selectively extracted from the water by an organic solvent whose residue is not toxic to X. malvacearum.

Whatever solute characteristics or methods are employed for further purification of the phytoalexin(s), extracts stored for greater than 1 week should not be used. Figures 14 and 15 as well as other HPLC profiles not shown, suggest that decomposition or chemical modification of

extraction components occurs under the storage conditions used (-10°C, under nitrogen). Figures 14 and 15 represent samples from the same preparation; samples for Figure 15 were chromatographed 14 days after those for Figure 14. The most striking differences between the two profiles, based on peak shape, retention time and eluting solvent, are the peaks in IXSP3'-3 and IXSP3'-5 (Figure 15) which correspond to peaks in IXSP3'-2 and IXSP3'-4 (Figure 14), respectively. It is apparent that the relative heights of the peaks in IXSP3'-3 and IXSP3'-5 of Figure 15 are inverted from the relative peak heights in IXSP3'-2 and IXSP3'-4 of Figure 14. The inversion in Figure 15, however, is not a proportional one; i.e., the amount of increased peak area in IXSP3'-5 is considerably greater than the amount of decreased peak area in IXSP3'-3. Since extinction coefficients of the components in these fractions, as well as their relative amounts, determine the total peak absorbance in each fraction, the source of the increased absorbance cannot be definitively assigned.

The structural nature of the phytoalexin(s) is not yet known; however, other phytoalexins from cotton as well as many from other sources (Table III) are polyphenols. Thus, it is likely that the partially purified phytoalexin(s) produced by Im 216 cotyledons in response to inoculation with X. malvacearum R4 is (are) also polyphenolic in nature. In further support of this idea is the fact that phenylalanine ammonia lyase (PAL) activity in many resistant hosts is stimulated as a result of pathogen invasion (70,28,40). Phenylalanine ammonia lyase diverts phenylalanine from protein synthesis to phenylpropanoid metabolism (e.g., flavonoids and lignins) by deamination to cinnamic acid. Hydroxylation of cinnamic acid leads to the formation of phenolic

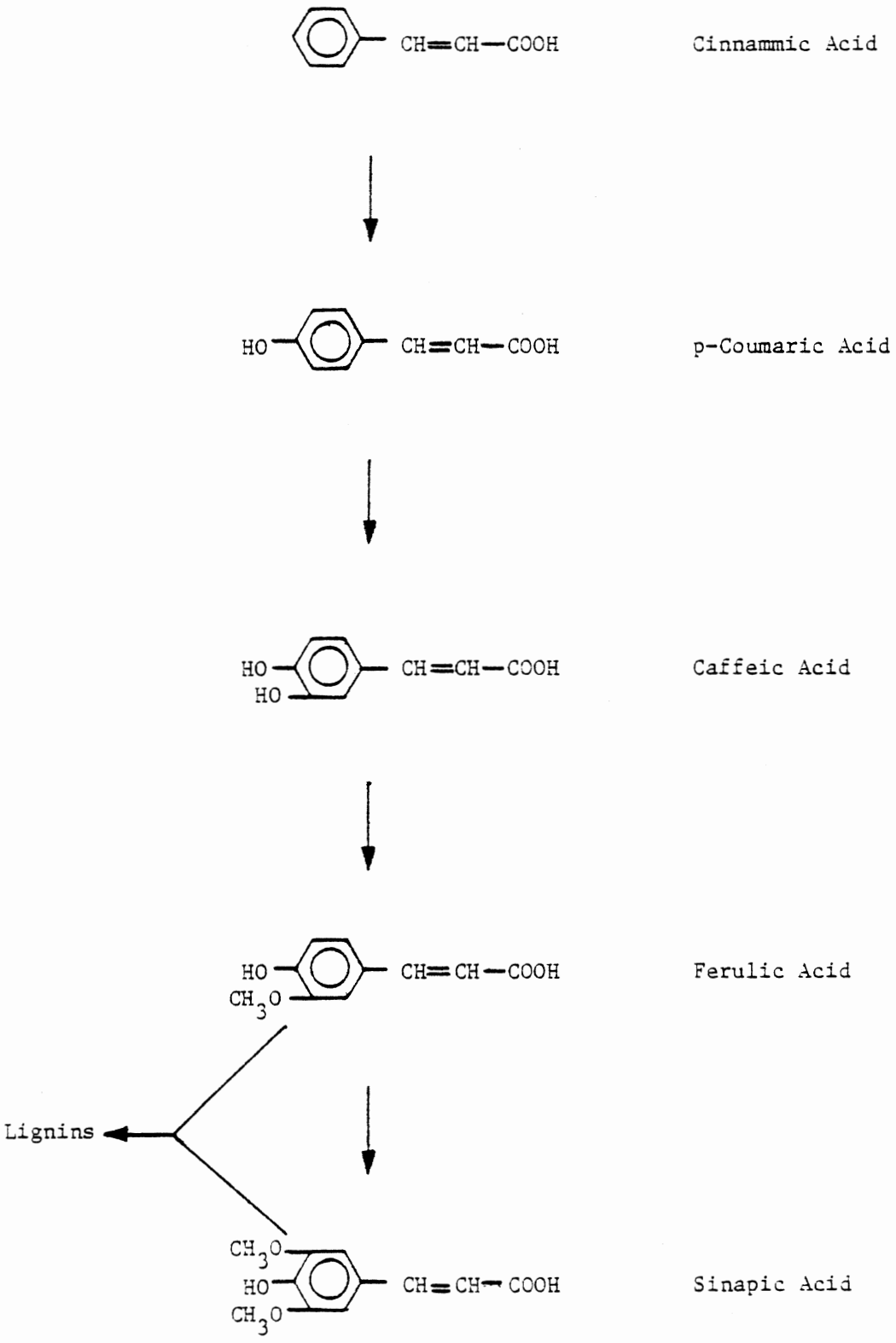
compounds such as p-coumaric acid, caffeic acid and others (Figure 16).

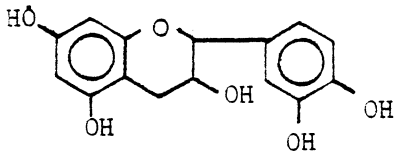
Since Venere (74) showed that peroxidase activity in Im 216 cotyledons increased following inoculation with X. malvacearum R3, it is also possible that the partially purified phytoalexin(s) is (are) the oxidation product(s) of one or more polyphenols. Consistent with this is the fact that polyphenoloxidase activity is also stimulated along with the PAL activity discussed earlier (70,28,40). This enzyme converts O-diphenols to O-diquinones.

Venere (84) has proposed that the polyphenol catechin is the peroxidase substrate in cotton because of catechin's abundance in cotton and because he found that the peroxidase-catalyzed oxidation products of catechin were toxic to X. malvacearum R3. Weinges, et al., (86) have isolated several peroxidase-catalyzed oxidation products of catechin and shown them to be polymers of catechin (Figure 17). Peroxidase has also been shown to be capable of dimerzing the cotton phytoalexin hemigossypol to gossypol (83).

Summary

Aqueous methanol intercellular fluid extracts from IX and IC cotyledons were complex mixtures of compounds, as evidenced by their TLC and HPLC patterns. Differential inhibitory activity existed in the IX extracts which indicated the presence of one or more phytoalexins. The phytoalexin(s) was (were) partially purified through SEP-PAK and high performance liquid chromatography (see Figure 18). The behavior of the phytoalexin(s) during purification indicates it (they) was (were) more polar than 2,7-dihydroxycadalene (another phytoalexin found in cotton). The phytoalexin material was also more polar than

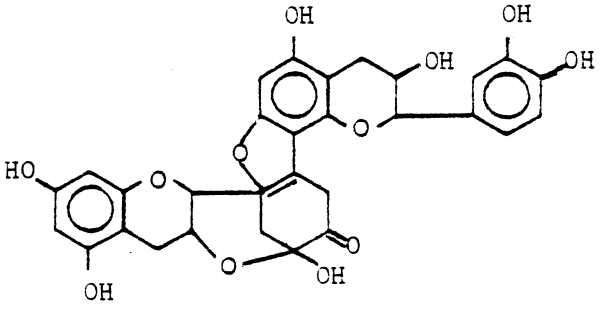

CH=CH—COOH
Cinnammic Acid
HO
CH=CH—COOH
p-Coumaric Acid
HO
HO
CH=CH—COOH
Caffeic Acid
HO
CH3O
CH=CH—COOH
Ferulic Acid
Lignins
CH3O
HO
CH3O
CH=CH—COOH
Sinapic Acid



Catechin

Horseradish Peroxidase,

H_2O_2



A Dicatechin

Inoculate Im 216 cotyledons with either
X. malvacearum (IX) or $CaCO_3$ (IC)

3 days

Extract intercellular solutes with 40% aqueous methanol

Remove methanol under N_2 at 30°C

Pass 2 ml methanol and two 5 ml volumes of H_2O through C_{18} SEP-PAKS

Pass aqueous extracts through pre-washed SEP-PAKS

Elute SEP-PAKS with 5 ml of each:
H_2O, 15% methanol and 35% methanol

(H_2O)	(35% methanol)	(15% methanol)
SP1'	SP3'	SP2'

HPLC on C_{18}, 1.0 ml/min

35% methanol (0-32 min), 50% methanol (32-44 min)

67% methanol (44-56 min) and 100% methanol (56-68 min)

Prepare and bioassay each fraction as

described in "MATERIALS AND METHODS"

catechin, a polyphenol abundant in cotton. For this reason and the work of Venere (84) and others (70,28,40,86), it was suggested the phytoalexin(s) was (were) the oxidation product(s) of catechin. Possible means of further purification were discussed.

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STUDIES ON THE RESISTANCE OF COTTON, *GOSSYPIUM HIRSUTUM*, TO BACTERIAL BLIGHT CAUSED BY *XANTHOMONAS MALVACEARUM*

II. Induced Resistance in Ac 44 Cotyledons

CHAPTER I

INTRODUCTION

The work presented in this section investigates the possibility of induced resistance to bacterial blight of cotton in Ac 44 cotyledons. Acala 44 is a breeding line of cotton that is susceptible to this disease, which is caused by Xanthomonas malvacearum. Infected Ac 44 leaves and cotyledons develop expanding water-soaked lesions which become chlorotic and within 2 to 3 weeks after infection become necrotic. It has been reported, however, for tobacco, bean and cucumber leaves that a resistance response can be induced in the normally susceptible hosts if the hosts are exposed to either live or heat-killed microorganisms, which are pathogenic or non-pathogenic, previous to inoculation with the virulent pathogen. The present work investigates whether a resistant response can be induced in Ac 44 cotyledons.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Induced resistance, also known as cross protection, has been described in several hosts (13,7,15,2,4,10,12,1). The two most studied hosts have been tobacco and cucumber leaves.

Lovrekovich and Forkas (11) demonstrated that inoculation of tobacco leaves with heat-killed cells of Ps. tabaci, the wildfire pathogen, produced a time-dependent protective response against inoculation with live cells of Ps. tabaci. Sequiera and Hill (14) showed that initial populations of live Ps. solanacearum (challenge inoculum) decreased rapidly after inoculation if the tobacco leaves had been inoculated 24 h previously with heat-killed Ps. solanacearum (protecting inoculum). Also, in protected tissue, tobacco cells were able to tolerate high levels of Ps. solanacearum with no development of disease symptoms or necroses (14). Fraser (5) found systemically induced resistance to tobacco mosaic virus (TMV) lesions in tobacco leaves. The lesions formed on upper leaves in response to a challenge inoculation with TMV (flavum) 10 days after inoculation of lower leaves were smaller and fewer than those formed on upper leaves of plants whose lower leaves did not receive the first inoculation. Although lesion size was smaller in leaves of protected plants, the viral numbers in these tissues were no different than those from unprotected plants.

Systemic induction of resistance has also been achieved in leaves

of eight susceptible cultivars of cucumber (7,3,9,8,6). Kuć and Richmond (8) showed that inoculation of a cotyledon or first true leaf of cucumber with Colletotrichum lagenarium systemically protected these plants against disease caused by subsequent challenge inoculations by this pathogen. Protection of leaf 2 was evident 96 h after inoculation of leaf 1. Each new leaf that formed for 4-5 weeks after inoculation of leaf 1 exhibited resistance to C. lagenarium.

Since protection against more than one pathogenic or non-pathogenic species can occur, induced resistance in normally susceptible hosts has a good potential for field use. This requires an easily applied agent that is capable of inducing cross protection in normally susceptible hosts.

The mechanism by which induced resistance occurs is not known. The non-specific relationship between the protecting organism and the challenging organism is not understood. Questions which need to be addressed are (1) can resistance inducers be isolated from the protecting organisms; (2) are host cells "sensitized" by the protecting organisms so that they can respond more quickly against the challenge organism; (3) is phytoalexin synthesis stimulated; and (4) does induced resistance occur naturally in the field?

The present work does not attempt to answer the above questions. Instead, it ascertains whether or not resistance to X. malvacearum R3 can be induced by either heat killed or live X. malvacearum R3 or by live X. vesicatoria (tomato pathogen). Variations in certain parameters are tested.

CHAPTER III

MATERIALS AND METHODS

Cotton Line

Acala 44 contains no major genes for resistance and is susceptible to all known races of X. malvacearum. Acid-delinted seeds were planted in a commercial mixture of peat moss and vermicalite (Jiffy Mix Plus, Jiffy Products of America, 250 Town Road, West Chicago, IL 60185) in either 20 cm x 32 cm flats (15 seeds/flat) and were grown in a growth chamber with a temperature cycle of 30°C days (12 h) and 16°C nights (12 h); or they were planted in 10 cm clay pots (2 seeds/pot) and were grown in a greenhouse maintained at 32°C during the day and 21°C during the night.

Growth Curves of Challenge Inoculum

The right halves (top view) of 3 week old cotyledons from Ac 44 greenhouse-grown plants were inoculated (20 cotyledons/treatment) with: calcium carbonate, 4.3×10^5 cfu/ml, 3.0×10^6 cfu/ml and 3.6×10^7 cfu/ml of live X. malvacearum; and 2.6×10^8 cfu/ml of X. malvacearum that had been heated to 100°C in a water bath for 10 min (heat killed). These were designated the protection inocula. Three days later the left halves of these cotyledons were inoculated (50 cotyledons/treatment) with 3.2×10^6 cfu/ml and 3.3×10^7 cfu/ml of live X. malvacearum

R3 (challenge inocula). At various times after the challenge inoculation, 3 discs were removed from the challenged side of one cotyledon and treated as described in the "Growth Curve" section of "MATERIALS AND METHODS" in section I of this thesis.

Measurement of Disease Development Rates

The right halves (top view) of 3 week old cotyledons from Ac 44 growth chamber-grown plants were inoculated (20 cotyledons/treatment) with: 2.2×10^5 cfu/ml and 3.7×10^7 cfu/ml of live X. malvacearum R3; ~ 10^8 cfu/ml of heat killed X. malvacearum R3; and 2.4×10^7 cfu/ml of X. vesicatoria. Two days later, the left halves of these cotyledons were spot inoculated (50 cotyledons/treatment) at four places with a 1 ml syringe that contained either 3.7×10^6 cfu/ml or 3.6×10^7 cfu/ml. At various time intervals after the challenge inoculation, the inoculated spots were examined for water soaking. The following grading scale was used to assess the degree of water soaking in each of the inoculated spots: 0 - no water soaking; 1 - 1-25% water soaked; 2 - 26-50% water soaked; 3 - 51-75% water soaked; and 4 - 76-100% water soaked. A weighted disease index (WDI) was calculated in the following manner:

$$\text{WDI} = \frac{4(\#\text{ of grade 4 spots}) + 3(\#\text{ of grade 3 spots}) + 2(\#\text{ of grade 2 spots}) + 1(\#\text{ of grade 1 spots})}{4(\#\text{ of total spots for that treatment})}$$

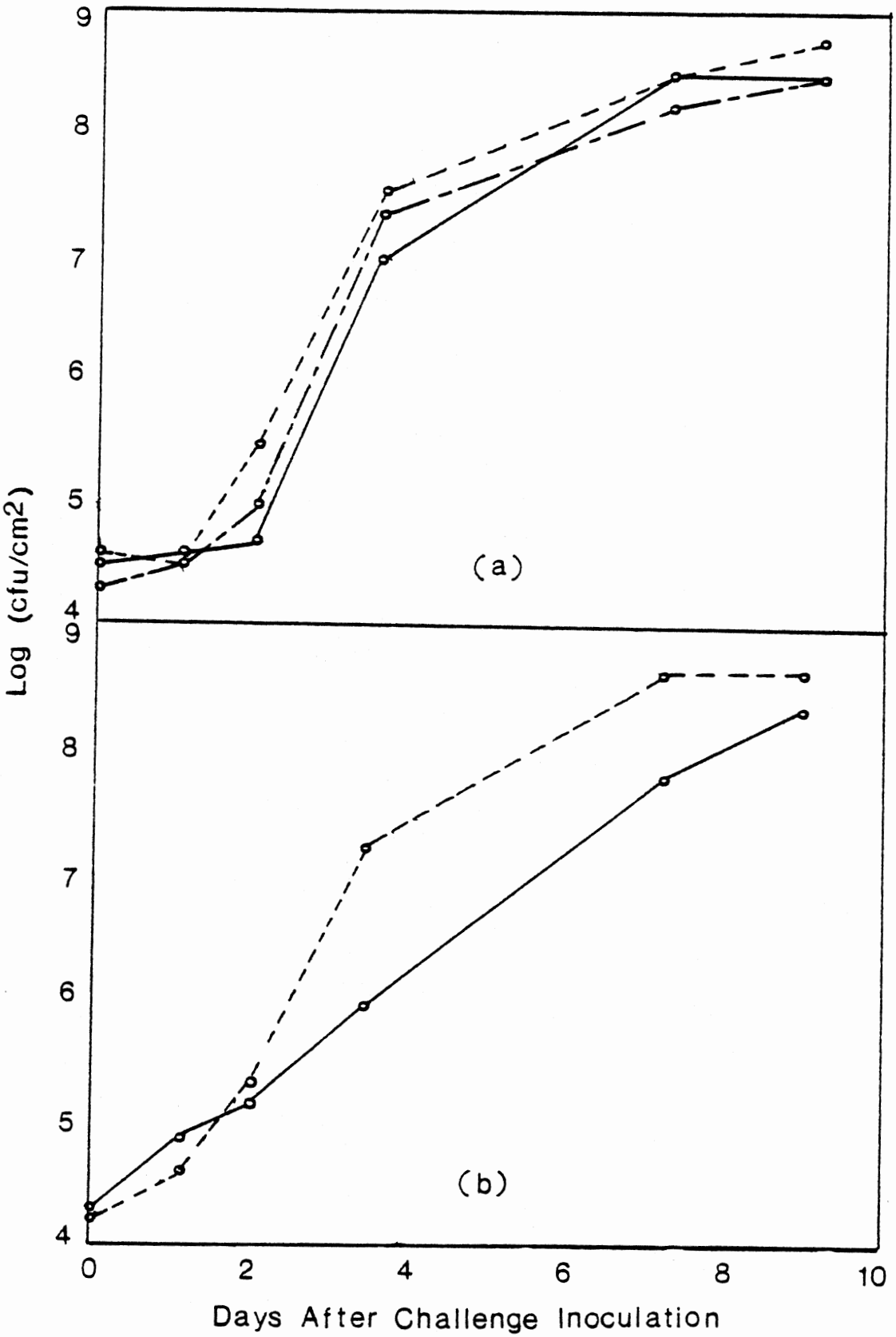
where the denominator represents the value if all spots were grade 4. Thus, the greater the WDI, the more the disease has developed; a WDI of 1.0 indicates all spots greater than 75% water soaked.

CHAPTER IV

RESULTS

Since growth of X. malvacearum in the immune host, Im 216, was inhibited 3 days after inoculation (Figure 1, Section I), expression of induced resistance in Ac 44 might manifest itself by inhibition of the growth of the challenging organism. For that reason, Ac 44 cotyledons were inoculated with calcium carbonate or with four levels of live X. malvacearum R3 (protection inoculum) and were challenged 3 days later with two levels of live X. malvacearum R3 as described in "MATERIALS AND METHODS"; growth of the challenge inouclum was monitored over a 7-day period. The results are shown in Figures 1 and 2 and in Table I.

Figures 1 and 2 show no growth inhibiton with any PrI/ChI combination other than the 3.6×10^7 cfu/ml/3.3×10^7 cfu/ml combination. The maximum level attained by X. malvacearum in cotyledons inoculated with this level was only 10^7 cfu/cm^2; all other combinations grew to between 10^8 and 10^9 cfu/cm^2, the normal ceiling level for X. malvacearum in Ac 44 cotyledons (see Figure 1, Section I). Table I shows that there were only three PrI/ChI combinations which induced inhibitory action toward the growth of the ChI: 3.6×10^7 cfu/ml/3.2×10^6 cfu/ml, 3.6×10^7 cfu/ml/3.3×10^7 cfu/ml and 2.6×10^8 cfu/ml heat killed/3.3×10^7 cfu/ml.


9
8
7
6
5
4
Log (cfu/cm2)
(a)
9
8
7
6
5
4
(b)
0
2
4
6
8
10
Days After Challenge Inoculation

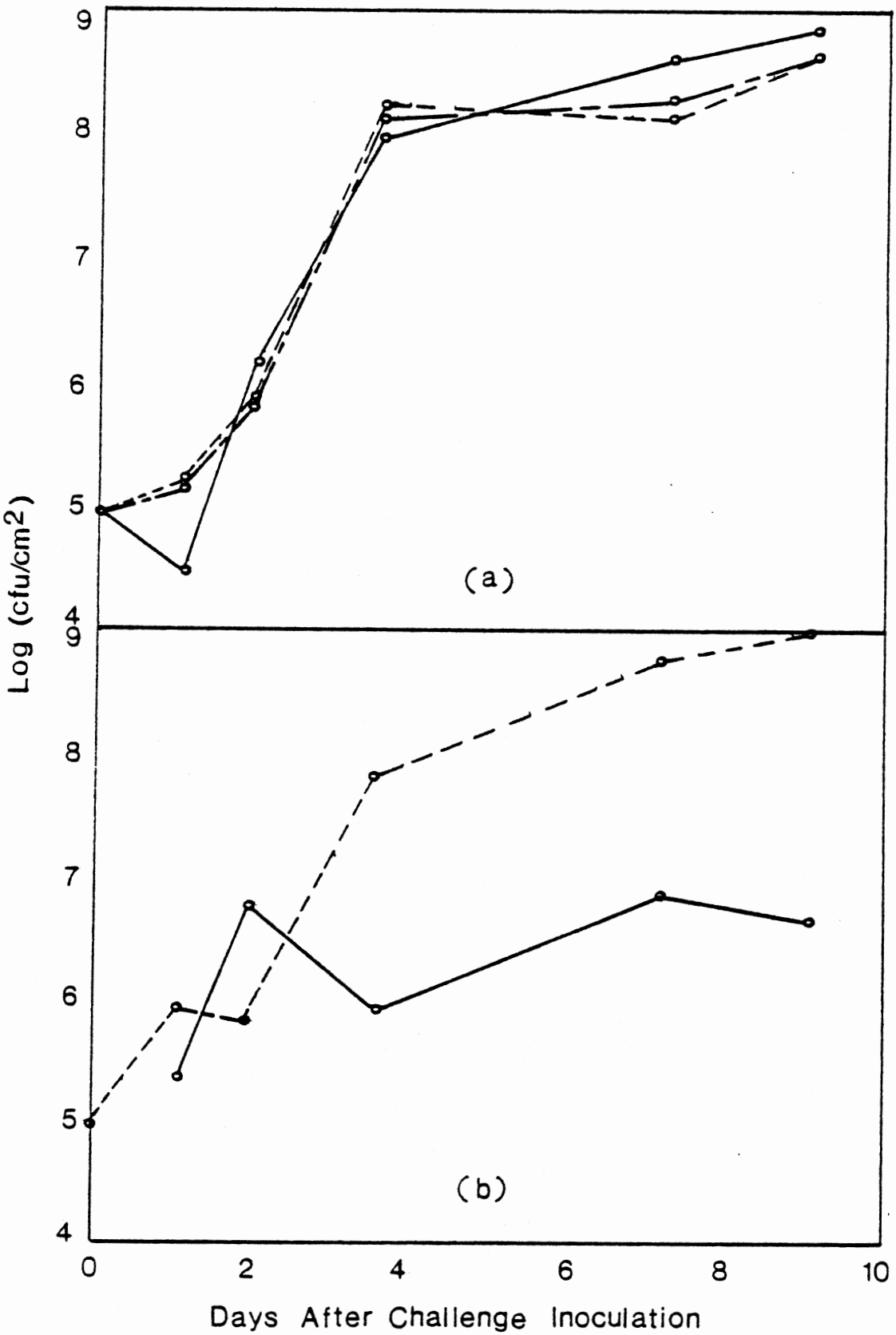

9
8
7
6
5
4
(a)
9
8
7
6
5
4
(b)
0
2
4
6
8
10
Log (cfu/cm2)
Days After Challenge Inoculation

TABLE I

LOGARITHMIC GROWTH DOUBLING TIMES OF CHI POPULATIONS IN Ac 44 COTYLEDONS--TAKEN FROM FIGURES 1 AND 2

PrI (cfu/ml)/ChI (cfu/ml)	ChI Doubling Time (t_D)[1] (h)
Calcium carbonate/3.2×10^6	1.8
$4.3 \times 10^5/3.2 \times 10^6$	1.8
$3.0 \times 10^6/3.2 \times 10^6$	1.8
$3.6 \times 10^7/3.2 \times 10^6$	1.9
2.6×10^8 heat killed/3.2×10^6	1.8
Calcium carbonate/3.3×10^7	1.8
$4.3 \times 10^5/3.3 \times 10^7$	1.8
$3.0 \times 10^6/3.3 \times 10^7$	1.8
$3.6 \times 10^7/3.3 \times 10^7$	2.0
2.6×10^8 heat killed/3.3×10^7	1.9

[1] t_D values were calculated from growth between 39 and 98 h.

Measurement of Disease Development Rate

Since only one PrI level induced resistance to only one ChI level in terms of growth inhibition, another resistance parameter was examined. Cotyledons were inoculated as described in "MATERIALS AND METHODS" with the following organisms as protecting agents: X. vesicatoria and various levels of live and heat-killed cells of X. malvacearum. Challenges with live X. malvacearum R3 were made on the non-inoculated half of each cotyledon 2 days after the protection inoculations. Disease symptom progression in the challenged cotyledon halves was followed by direct visual observation and evaluated by assigning a weighted disease index (WDI) value for each observation (see "MATERIALS AND METHODS"). The results are shown in Figure 3 and Table II.

Figure 3(a) and Table II show that disease symptoms form at the same rate in cotyledons protected with 2.2×10^5 cfu/ml as with the control cotyledons (no PrI). Those protected with 3.7×10^7 cfu/ml, however, experienced a definite initial lag in disease development which was followed by an accelerated rate of disease development relative to the control cotyledons. The net disease development rate equaled that of the control. Figure 3(b) and Table II showed that none of the inocula tested protected against disease development caused by 3.6×10^7 cfu/ml.

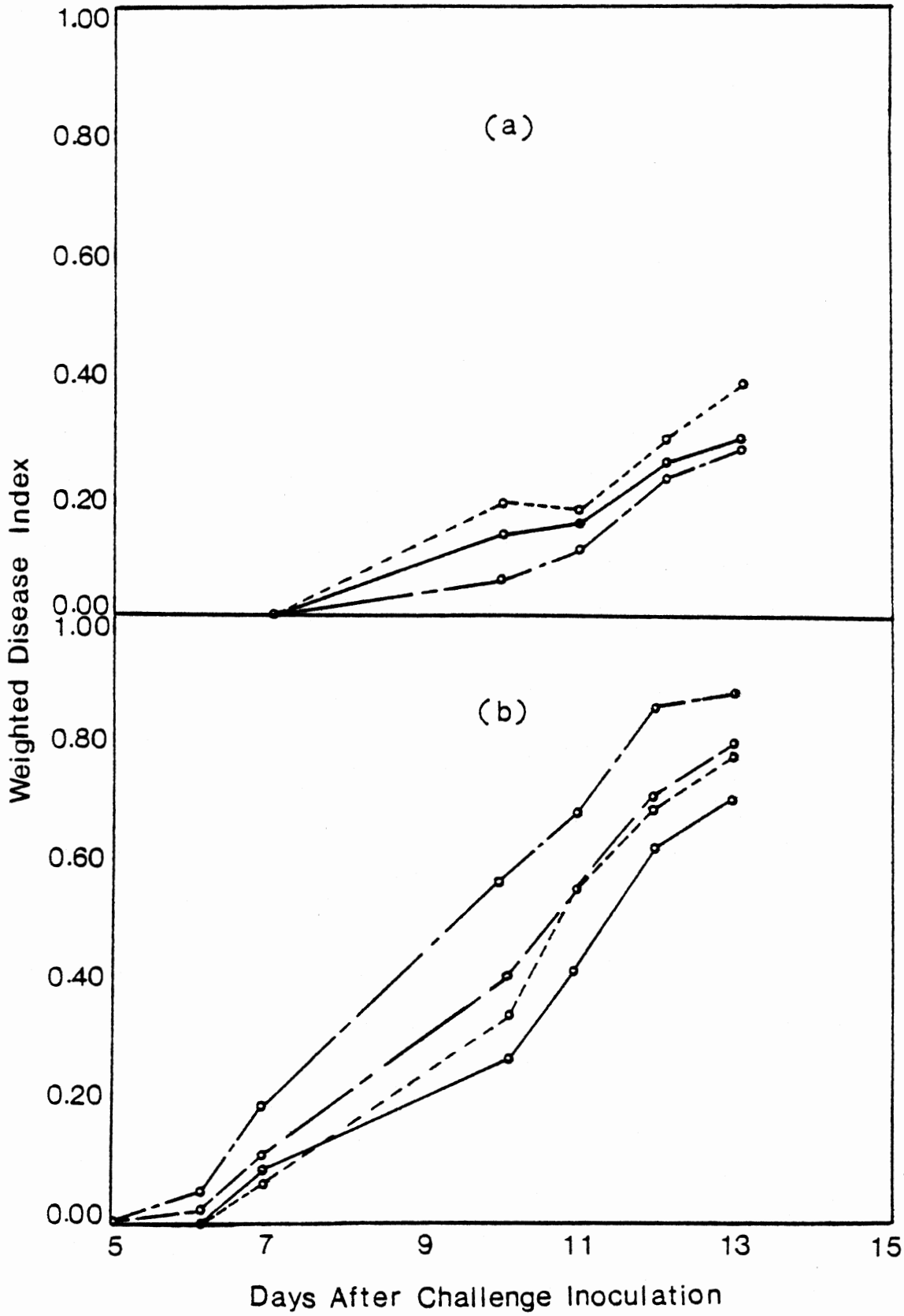

1.00
0.80
0.60
0.40
0.20
0.00
(a)
1.00
0.80
0.60
0.40
0.20
0.00
(b)
Weighted Disease Index
5
7
9
11
13
15
Days After Challenge Inoculation

TABLE II

RATE OF DISEASE DEVELOPMENT IN PROTECTED AND UNPROTECTED TISSUE--TAKEN FROM FIGURE 3

PrI (cfu/ml)/ChI (cfu/ml)	Disease Development Rate[1] (ΔWDI/Day)
Nothing/3.7×10^6	0.050
$2.2 \times 10^5/3.7 \times 10^6$	0.060
$3.7 \times 10^7/3.7 \times 10^6$	0.025[2] 0.095[3] 0.049[4]
Nothing/3.6×10^7	0.097
$3.7 \times 10^7/3.6 \times 10^7$	0.11
$\sim 10^8$ heat killed/3.6×10^7	0.12
2.4×10^7 X. vesicatoria/3.6×10^7	0.10

[1]Slopes were calculated from linear least squares-generated lines.

[2]Slope was calculated from data at 7, 10 and 11 days.

[3]Slope was calculated from data at 11, 12 and 13 days.

[4]Slope was calculated from data at 7 through 13 days.

CHAPTER V

DISCUSSION

Table I shows that three PrI/ChI combinations induced slower growth of their ChI populations (longer t_D) than the controls. The protecting inoculum for two of these combinations was 3.6×10^7 cfu/ml. In addition, Figure 2 showed that inoculation with 3.6×10^7 cfu/ml resulted in a lower than normal maximum population of bacteria from a 3.3×10^7 cfu/ml challenge.

These results suggest that the minimum concentration of live X. malvacearum required to induce resistance to live X. malvacearum in Ac 44 cotyledons is between 3.7×10^6 cfu/ml and 3.6×10^7 cfu/ml. In further support of this is the initial lag in disease development in cotyledons protected with 3.7×10^7 cfu/ml and challenged 2 days later with 3.7×10^6 cfu/ml (Figure 3(a)). The reason for this minimum protection concentration of X. malvacearum might be that a critical (minimum) number of Ac 44 cells must contact or be close to cells of X. malvacearum for resistance to be induced.

While in the lag phase of disease development (Days 7-11), the disease development rate for the cotyledons protected with 3.7×10^7 cfu/ml and challenged with 3.7×10^6 cfu/ml was half that of the control; whereas, the rate from days 11-13 was almost twice that of the control (Table II). The overall rate, however, was equal to the disease development rate in control cotyledons. Thus, the resistance

that was induced by the 3.7×10^7 cfu/ml level of X. malvacearum was overcome 4 days after the cotyledons were challenged. This may reflect an insufficient time interval between the protection and challenge inoculations to allow for completion of the induced resistance mechanism.

Concluding Remarks

More experiments are needed to ascertain, for certain, if a resistance response can be induced in Ac 44 cotyledons and upper leaves. Some protection inocula which should be tested are: increasing levels of live X. malvacearum (several races) starting at 4.0×10^7 cfu/ml; X. malvacearum cells which have been killed through a treatment other than boiling for 10 minutes; culture filtrate; and non-pathogens. In addition, various time intervals between the protection and challenge inoculations should be tested.

Summary

Induced resistance to X. malvacearum R3 in Ac 44 cotyledons was investigated by following growth of the challenge populations and the rate of disease development. By each criterion, a minimum concentration of live X. malvacearum R3 required to protect against subsequent challenge by X. malvacearum R3 2 or 3 days later was between 3.7×10^6 cfu/ml and 3.6×10^7 cfu/ml. Only growth of the 3.3×10^7 cfu/ml challenge inoculum was inhibited in protected tissue as evidenced by the increased doubling time (Table I) and the lower maximum growth level (Figure 2). Cotyledons protected with 3.7×10^7 cfu/ml and challenged with 3.7×10^6 cfu/ml 2 days later experienced a lag in disease development as compared to unprotected cotyledons. By the two

criteria tested, it seems at least partial resistance can be induced in Ac 44 cotyledons.

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VITA

Steve John McFaul

Candidate for the Degree of

Doctor of Philosophy

Thesis: STUDIES ON THE RESISTANCE OF COTTON, GOSSYPIUM HIRSUTUM L., TO BACTERIAL BLIGHT CAUSED BY XANTHOMONAS MALVACEARUM: I. PARTIAL PURIFICATION OF A PHYTOALEXIN PRODUCED IN Im 216 COTYLEDONS; II. INDUCED RESISTANCE IN Ac 44 COTYLEDONS

Major Field: Biochemistry

Biographical:

Personal Data: Born in Evanston, Illinois, December 29, 1951, the son of Mr. and Mrs. D. B. McFaul.

Education: Graduated from Notre Dame High School, Niles, Illinois, in June, 1969; received Bachelor of Science degree in Chemistry from the College of Racine in May, 1973; received Master of Science degree in Biochemistry from Clemson University in May, 1976; completed requirements for the Doctor of Philosophy degree in Biochemistry from Oklahoma State University in July, 1981.

Professional Experience: Graduate teaching assistant, Chemistry Department, Oklahoma State University, 1976-1977; graduate teaching assistant, Biochemistry Department, Oklahoma State University, 1978.